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Osmo- and Iono-Regulation during Early Developmental Stages of Teleosts

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Contents

General Introduction

It is well known that teleosts are able to maintain the osmolarity and ion concentrations of their body fluid at constant levels different from external environments. Seawater (SW) teleosts continuously lose water and gain ions mainly through the respiratory epithelia of the gills, which occupy the most area of the body surface. In order to compensate for the water loss, they drink SW and absorb both water and ions along the intestine. The excess Na^+ and Cl^- are excreted from specialized, ion-transporting cells named "chloride cells" in the gill epithelium. On the other hand, freshwater (FW) teleosts face the problem of ion loss and water influx. They scarcely drink water, and produce a large amount of hypotonic urine in the kidney. The loss of ions is compensated by active uptake of ions from dilute environments across the gills. Thus, the gills, kidney and intestine are important osmoregulatory organs in teleosts. Although embryos and larvae of teleosts lack fully-developed osmoregulatory organs, they are also able to maintain the internal osmolarity and ion concentrations; they seem to possess some mechanisms to maintain their internal hydromineral balance, which are different from those of adult fish (Alderdice 1988). However, less information is available on the mechanism of osmotic and ionic regulation in embryos and larvae than that in adult fish. In the present study, I have attempted to reveal osmo- and iono-regulation in the early developmental stages of teleosts.

In the past, many studies have been carried out on the hormonal control of osmotic and ionic regulation of teleosts. It is well established that prolactin is a hormone essential for FW adaptation, and cortisol and growth hormone for SW adaptation (Hirano 1986; Hirano *et al.* 1987; Bern and Madsen 1992; Wendelaar Bonga 1993). However, most of these studies have been done using adult fish of euryhaline teleosts such as salmonids, and little is known on mechanisms of osmotic and

ionic regulation in embryos and larvae, especially in those of marine teleosts which occupy a large proportion of teleost species. Some marine teleosts hatch from small pelagic eggs, spend planktonic life in offshore areas, and migrate to inshore habitats during metamorphosis from larvae to juveniles. Japanese flounder (*Paralichthys olivaceus*) is one of those marine teleosts which migrate from offshore to near-shore areas during metamorphosis in nature (Minami 1982), and occasionally encounter low-salinity waters. In Chapter 1, I selected Japanese flounder as one of the representatives of marine teleost species, and examined the involvement of prolactin, cortisol and thyroid hormones in low-salinity adaptation during early developmental stages.

Chloride cells, often referred to as ionocytes or mitochondria-rich cells, in the gill epithelium are the important site for ion excretion in SW teleosts. In embryos and larvae of several teleosts, chloride cells have been detected in the epithelia covering the yolk and body, and these extrabranchial chloride cells are considered to be the site for ionic regulation during early developmental stages without functional gills (Shelbourne 1957; Lasker and Threadgold, 1968; Hwang and Hirano 1985; Alderdice 1988; Hwang 1989, 1990; Ayson *et al*. 1994; Hwang *et al*. 1994; Kaneko *et al*. 1995; Shiraishi *et al*. 1997; Sasai *et al*. 1998). Although one can easily expect that these extrabranchial chloride cells as the major site for ion secretion are taken over by branchial chloride cells as fish grow, the spatial shift of chloride cell distribution from the yolk-sac membrane and body skin to gills and their developmental sequence have not been demonstrated. In Chapter 2, I examined the development of both cutaneous and branchial chloride cells during early developmental stages of Japanese flounder by whole-mount immunocytochemistry with an antiserum specific for Na^+,K^+-ATPase, a key enzyme of ion transport in chloride cells.

It has certainly great advantages to employ Japanese flounder as an experimental model for osmotic and ionic regulation in early life stages of fish, not only because of their prominent

metamorphosis from larvae to juveniles, but also because of their commercial importance. Nevertheless, Japanese flounder is not adaptable to pure FW, and thus does not serve as a model for FW adaptation. In contrast, Mozambique tilapia (*Oreochromis mossambicus*) possess strong euryhalinity throughout their life history: they can mature and breed in both FW and SW, and the embryos can survive direct transfer from FW to SW and *vice versa*. Thus, the tilapia is another suitable experimental model for studies on osmotic and ionic regulation in the early developmental stages. Recent studies have proved that tilapia embryos and larvae possess numerous chloride cells in the epithelia covering the large yolk, and that chloride cells in SW embryos and larvae are larger than those in FW (Ayson *et al*. 1994; Shiraishi *et al*. 1997). These studies suggest the occurrence of FW- and SW-type chloride cells in the yolk-sac membrane of tilapia embryos and larvae, as is the case with gill chloride cells in adult fish (Pisam and Rambourg 1991; Uchida *et al*. 1996; Sasai *et al*. 1999; Hirai *et al*. 1999). The strong euryhalinity of tilapia embryos and larvae seem to be attributed to chloride cells in the yolk-sac membrane, which may function as the sites for ion uptake and secretion in FW and SW, respectively. From a physiological and morphological point of view, it is of great interest to examine whether chloride cells are replaced with newly-differentiated cells of a different function when transferred from FW to SW or from SW to FW, or the same chloride cells function in both FW and SW. In Chapter 3, I carried out an *in vivo* follow-up observation on individual chloride cells in the yolk-sac membrane of tilapia embryo and larvae transferred from FW to SW. It is difficult to observe time-course changes in gill chloride cells of adult fish because of the complex structure of the gills. The simple flat structure of the yolk-sac membrane and the combination of vital staining of chloride cells and confocal laser scanning microscopy allowed me to observe sequential changes in individual chloride cells.

Freshwater teleosts take up ions from dilute environments across the gills, compensating for

the constant loss of ions by diffusion (Lin and Randall 1995; Goss *et al.* 1995; Flik *et al.* 1995). However, the mechanism of ion uptake in the gills of FW teleosts is less certain than the salt-secreting mechanism in SW. It is suggested that the branchial Na^+ uptake occurs through a Na^+ channel electrically coupled with a vacuolar-type H^+-ATPase (V-ATPase), which generates a favorable electrochemical gradient for passive electrodiffusion of Na^+ through a Na^+ channel (Lin and Randall 1995). However, cellular localization of V-ATPase is still controversial in teleosts: previous studies using rainbow trout (*Oncorhynchus mykiss*) gills reported that V-ATPase immunoreactivity is distributed in both chloride and respiratory pavement cells (Lin *et al.* 1994) or restricted to pavement cells (Sullivan *et al.* 1995). Since little information is available on ion uptake mechanisms during early developmental stages, I examined changes in whole-body Na^+ contents and cellular localization of V-ATPase of FW tilapia embryos and larvae in Chapter 4. The results suggest that Na^+ uptake occurs in pavement cells in the yolk-sac membrane of FW-adapted tilapia during early life stages, when functional gills are lacking.

Chapter 1: Developmental Changes in Low-Salinity Tolerance and Responses of Prolactin, Cortisol and Thyroid hormones to Low-Salinity Environment in Larvae and Juveniles of Japanese Flounder, *Paralichthys olivaceus*

ABSTRACT—In Japanese flounder (*Paralichthys olivaceus*), metamorphic period involves not only transformation from larva to juvenile but also migration from offshore areas to estuaries. In the present study, the role of endocrine systems in low-salinity adaptation was examined during early development and metamorphosis of the flounder. Survival rate 48 h after transfer to 1/8 SW was relatively high in yolk-sac larvae, decreased gradually to 0% at premetamorphosis, and increased to 100% at metamorphic climax. The ratio of prolactin (PRL) -immunoreactive part to whole pituitary increased gradually during larval stages and reached a constant level during metamorphosis. When the larvae at premetamorphosis and metamorphic climax and the benthic juveniles were transferred from SW to 1/4 SW, PRL-immunoreactive part increased significantly 48 h after the transfer at all stages examined. Whole-body concentration of cortisol was measured with a modified extraction method which is much robuster to lipid-rich sample than the ordinary method, but no significant difference was observed after the transfer. Whole-body concentrations of thyroid hormones decreased slightly but significantly at premetamorphosis and metamorphic climax. These results suggest possible involvement of PRL and thyroid hormones in low-salinity adaptation of the flounder during metamorphosis and inshore migration.

INTRODUCTION

In teleosts, it is well established that prolactin (PRL) is a hormone essential for freshwater

(FW) adaptation, and cortisol for seawater (SW) (Evans 1979; Henderson and Garland 1981; Hirano 1986; Hirano *et al.* 1987). However, most of the studies have been carried out using adult euryhaline fishes, and little is known on the endocrine control of osmoregulation in larvae or juveniles, especially of marine teleosts. Generally, larvae of marine teleosts hatching from small pelagic eggs spend planktonic life in offshore areas. The larvae of some species migrate to inshore habitats during metamorphosis, and this migration is named "inshore migration" (Creutzberg *et al.* 1978; Tanaka 1991). Japanese flounder (*Paralichthys olivaceus*) also migrates from offshore areas to estuaries during metamorphosis in nature (Minami 1982), and occasionally encounters low-salinity water. Although endocrine control of flounder metamorphosis has been extensively studied (see a review by Inui *et al.* 1994), no information is available on the hormonal control of osmoregulation during their early development and metamorphosis. In the present study, changes in low-salinity tolerance and PRL-cell volume were examined during flounder development, as well as the responsiveness of PRL, cortisol, and thyroid hormones to low-salinity environment.

MATERIALS AND METHODS

Fish

Naturally spawned eggs of Japanese flounder (*Paralichthys olivaceus*) were collected from the brood-stock tank in the Fisheries Research Station of Kyoto University. Larvae and juveniles were reared in a polycarbonate tank (500 l) with running SW. Water temperature was maintained at 18°C, and salinities ranged between 30.6 and 32.2 ppt. They were initially fed rotifers (*Brachionus plicatilis*) cultivated with *Nannochrolopsis* sp. and ω-Yeast (Kyowa Hakko Kogyo, Japan), and later brine shrimp (*Artemia* spp.) nauplii enriched with Ester-85 (Nippon Chemical Feed, Japan).

Transfer to low-salinity water

Forty fish at different developmental stages were transferred from the stocking tank (SW: 32ppt) to 2-l beakers containing FW (0 ppt), 1/8-diluted SW (4 ppt), 1/4-diluted SW (8 ppt), 1/2-diluted SW (16 ppt) or SW (32 ppt) using a pipette, and survival rates were examined 48 h after the transfer. Water temperature was maintained at 18°C. Photoperiod was 13 L (6:00 - 19:00) and fish were transferred at 17:00. They were not fed during the experiment.

Responsiveness of hormones to low-salinity water was examined at three developmental stages: premetamorphosis (18 days after hatching, 6.5 - 7.0 mm body length, body symmetrical, D stage); metamorphic climax (33 days after hatching, 9.5 - 10.2 mm, right eye at dorsal ridge, H stage); juvenile (49 days after hatching, 16.5 - 18.0 mm, benthic, I_4 stage). The developmental stages were described by Minami (1982) and Goto *et al.* (1989). They were transferred from the stocking tank to 2-l beakers containing 1/4 SW or SW (control), and fed *Artemia* nauplii once a day. Samples taken at 0 and 48 h after the transfer were fixed in Bouin's solution or preserved at -30°C. The samples for cortisol measurement were also taken at 1, 3, 6, 12, and 24 h after the transfer. Each frozen sample (about 100 mg) consisted of 30, 7 and 3 individuals at premetamorphosis, metamorphic climax and juvenile, respectively.

Prolactin immunohistochemistry

Larvae and juveniles at various developmental stages were fixed in Bouin's solution for 2-6 h at 4°C, dehydrated through graded ethanol, cleared in xylene, and embedded in Parahisto (Nacalai Tesque, Japan). Serial sagittal sections were made at 4 μm thickness and mounted on slides. The sections were deparaffinized, rehydrated, and stained by the peroxidase-antiperoxidase (PAP)

method (Sternberger *et al.* 1970) using commercial reagents (Dako, Denmark). The sections were incubated sequentially with: (1) 3% H_2O_2 for 10 min, (2) normal goat serum diluted 1:20 with 0.01 M phosphate-buffered saline (PBS, pH 7.2) for 30 min, (3) rabbit anti-tilapia PRL_{177} serum (Ayson *et al.* 1993) diluted 1:4000 for 20 h at 4°C, (4) goat anti-rabbit IgG serum diluted 1:100 for 60 min, (5) rabbit PAP diluted 1:100 for 80 min, (6) 0.02% 3, 3'-diaminobenzidine tetrahydrochloride containing 0.005% H_2O_2 for 5 min. The sections were then dehydrated, cleared, and mounted.

In all serial sections containing a portion of the pituitary, outlines of both PRL cells and the whole pituitary were traced on paper with a camera lucida. The images were digitized with a flat bed scanner (Sharp, Japan) and areas were measured on an Apple Macintosh computer using the public domain NIH Image program (available on the Internet at http://rsb.info.nih.gov/nih-image/). Total volumes of PRL cells and of the pituitary were calculated from the areas of the each section and the thickness (4 μm), and the percentage of PRL-cell volume to pituitary volume was calculated as a quantitative criterion of PRL-cell activity (Kimura and Tanaka 1991; Tanaka *et al.* 1995).

Cortisol and thyroid hormone measurements

In cortisol radioimmunoassay (RIA), we encountered extremely high level of nonspecific binding (NSB) by the use of a standard extraction method for tissue cortisol (de Jesus *et al.* 1991). This extraction method without the steps to remove lipids seems to be applicable only for lipid-less tissue or tissue free from some specific fatty acids. In the recent sea-farming scene, live feeds for marine fish larvae are enriched with n-3 highly unsaturated fatty acids (see a review by Watanabe and Kiron 1994). Consequently, tissues of the reared marine fish larvae would contain high quantity of lipids. Since lipids strongly interfere with steroid RIAs (Rash *et al.* 1979; Rash *et al.* 1980), we established and validated an extraction method using tetrachloromethane to eliminate lipid

contamination.

The frozen samples weighing 100-400 mg were homogenized in five-fold volume of ice-cold PBS using Polytrone homogenizer on a 10S blade (Kinematica, Switzerland). The homogenate (300 μl) was extracted twice with 3 ml of diethyl ether by mixing vigorously for 2 min. After freezing at -80°C, the ether layer was collected by decantation and dried at room temperature. To reconstitute the dry residue, 300 μl of tetrachloromethane was added and mixed for 4 min. PBS containing 0.1% gelatin (300 μl) was then added, and mixed for 2 min. After centrifugation (3000 rpm, 10 min, 4°C), the aqueous layer was divided into four 50-μl aliquots. The two aliquots were used for the cortisol RIA as described by Takahashi *et al.* (1985), and the other two for nonspecific binding (NSB) measurement.

By using the standard extraction method for tissue cortisol, NSB of extracts from metamorphic-climax larvae was 83 ± 9% (NSB / B_0, mean ± standard deviation, n = 7). By the present method with tetrachloromethane extraction, NSB was reduced to 1.0 ± 2.7% (n = 20). Figure 1 shows the competitive binding curves for the tetrachloromethane-washed extracts of the larvae in metamorphic-climax.

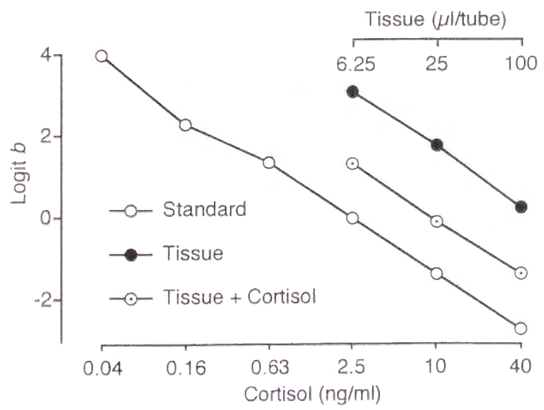


Fig. 1. Competitive binding curves for tetrachloromethane-washed extracts of metamorphic-climax larvae of the flounder. Each point represents the average of duplicate determinations. Logit $b = \log_e \{b / (100 - b)\}$. b = Bound / B_0 (%).

The dilution curve for larval extracts was parallel to the cortisol standard. The curve for the larval extracts added to cortisol was also parallel to the cortisol standard, and the recovery of cortisol was 96.5 ± 15.9% (n = 5).

Thyroxine (T4) and triiodothyronine (T3) were extracted and measured by RIAs as described by Tagawa and Hirano (1989).

Statistics

Significant differences in the % PRL-cell volume / pituitary volume and in the levels of thyroid hormones between the group transferred to 1/4 SW and the control group were tested by the Mann-Whitney test. Significant difference in the level of cortisol was examined by two-way ANOVA with repetition (salinity x time), as homogeneity of variance was established.

RESULTS

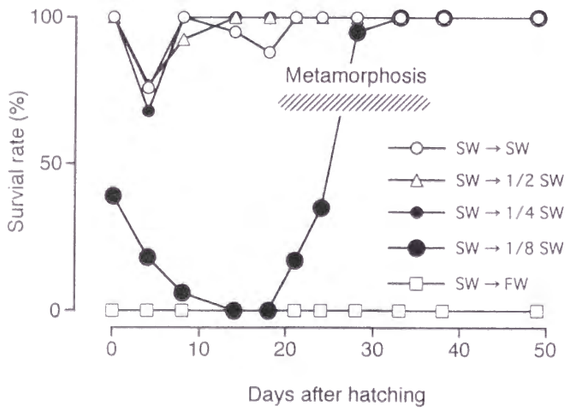


Fig. 2. Developmental changes in survival rates 48 h after the transfer. Each point represents the survival rate of 40 individuals.

Figure 2 shows the developmental changes in survival rates at 48 h after the transfer.Survival rates of the groups transferred to SW, 1/2 SW and 1/4 SW were more than 90% at most of the stages except for the larvae 4 days after hatching, showing about 70% survival rate even for the larvae maintained in SW. Survival rate of the group transferred to 1/8 SW was relatively high (40%) in yolk-sac larvae, decreased gradually to 0% in premetamorphic larvae (14 days after hatching), started increasing at the beginning of metamorphosis (21 days after hatching), and reached 100% at metamorphic climax. All the larvae and juveniles transferred to FW died within 24h at all stages.

PRL-immunoreactive cells were detected in the rostral pars distalis of the pituitary at all stages, except for the larvae just after hatching, in which no positive stain was observed. Figure 3 shows the developmental changes in the % PRL-cell volume / pituitary volume of the larvae and juveniles reared in SW. PRL-cell volume increased gradually during larval stages, and reached a constant level of 10% in the middle of metamorphosis (28 days after hatching).

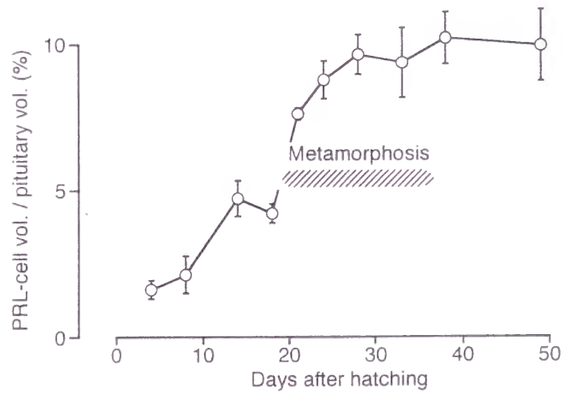


Fig. 3. Developmental changes in the percentage of PRL-cell volume to pituitary volume. Vertical bars represent standard errors of the means of 4 individuals.

Since 1/4 SW was the lowest salinity in which most of the larvae of all stages survived for 48 h, effects of low-salinity environment on hormone levels were examined by transferring the larvae at premetamorphosis (18 days after hatching) and metamorphic climax (33 days after hatching) and benthic juvenile (49 days after hatching) to 1/4 SW. Some larvae and juveniles were transferred to SW as controls. No mortality was observed in both groups during the transfer

experiments. Figure 4 shows PRL cells in the pituitary of metamorphic-climax larvae after the transfer, indicating that PRL-cell area of larvae transferred to 1/4 SW was larger than that to SW (control). Percent PRL-cell volume / pituitary volume in the groups transferred to 1/4 SW was significantly greater (Mann-Whitney test, $P < 0.05$) than those in the control groups at all three stages (Fig. 5).

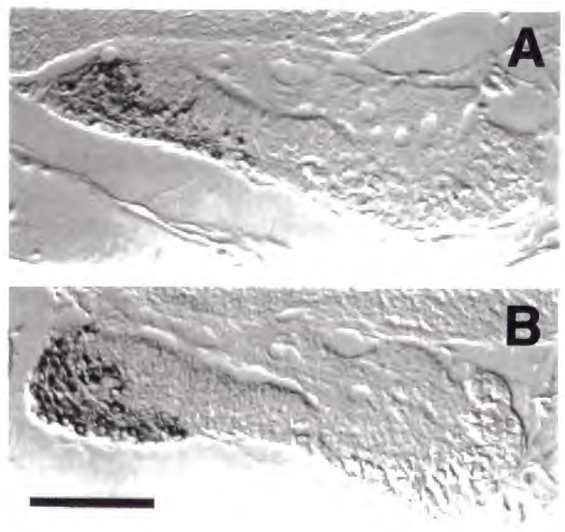


Fig. 4. PRL cells in the pituitary of flounder larvae at metamorphic-climax transferred from SW to SW (**A**) and from SW to 1/4 SW (**B**). The bar indicates 50 μm.

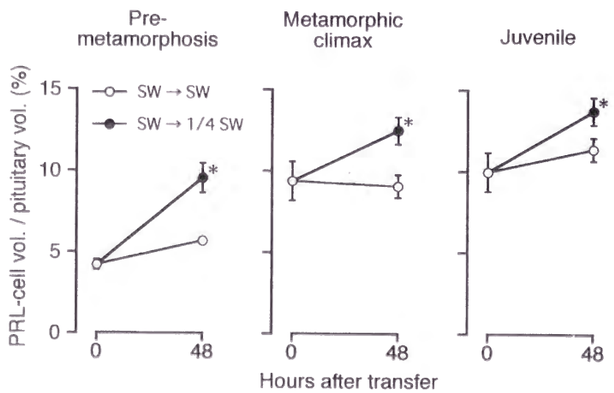


Fig. 5. Changes in the percentage of PRL-cell volume to pituitary volume after the transfer. Vertical bars represent standard errors of the means of 4 individuals. *Significantly different ($P < 0.05$) from the control by the Mann-Whitney test.

Figure 6 shows the changes in whole-body concentration of cortisol after the transfer. Two-way ANOVA showed a significant effect of time on cortisol concentration, but no significant effect

of salinity and a salinity x time interaction at all three stages. At each developmental stages, cortisol concentration reached the highest level 12 h after the transfer and then decreased. In metamorphic-climax larvae, cortisol concentration was relatively high at 0 h, decreased 1-3 h after the transfer, and increased later toward the highest level at 12 h.

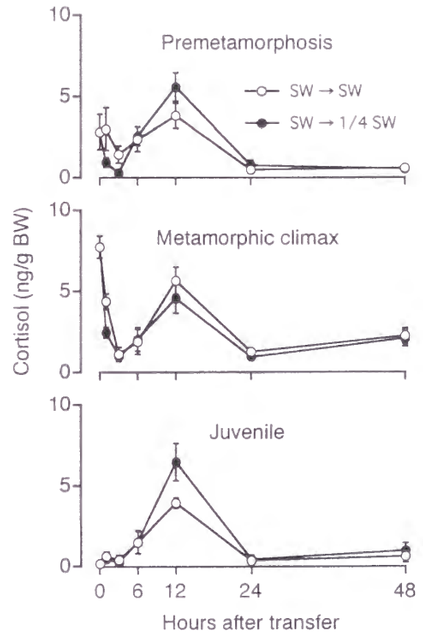


Fig. 6. Changes in the whole-body concentration of cortisol after the transfer. Vertical bars represent standard errors of the means of 4 pooled samples.

Figure 7 shows the changes in whole-body concentrations of thyroid hormones after the transfer. T4 concentration in the group transferred to 1/4 SW was significantly lower (Mann-Whitney test, $P < 0.05$) than that in the control group at premetamorphosis and metamorphic climax. T3 concentration was significantly lower (Mann-Whitney test, $P < 0.05$) in the larvae transferred to 1/4 SW than that in the control group at metamorphic climax.

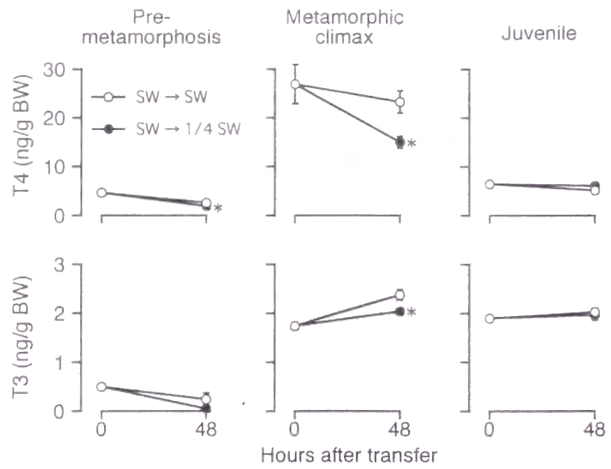


Fig. 7. Changes in the whole-body concentrations of T4 and T3 after the transfer. Vertical bars represent standard errors of the means of pooled samples (premetamorphosis, metamorphic climax: n = 4; juvenile: n = 5). *Significantly different ($P < 0.05$) from the control by the Mann-Whitney test.

DISCUSSION

A significant number of marine teleosts utilize estuaries as their nursery ground in their early life history, and inevitably encounter brackish water. Japanese flounder, one of the most important species for aquaculture in Japan, spawn in offshore areas of the sea. After spending pelagic life, flounder larvae undergo metamorphosis, migrate to estuaries, and occasionally encounter low-salinity water. In the present study, development of low-salinity tolerance of the flounder was clearly demonstrated in the group transferred to 1/8 SW, even though flounder larvae and juveniles would not be exposed to such a hypotonic environment in nature. In all groups, relatively high mortality was observed at 4 days after hatching when yolk was completely absorbed and feeding started. This stage is generally regarded as "critical period" of early life history in teleosts (Fabre-Demergue and Biétrix 1897; Minami 1994). An increase in low-salinity tolerance was observed during metamorphosis (21-28 days after hatching), coinciding with ecologically-observed migration to estuaries (Minami 1982; Tanaka *et al*. 1989).

PRL-cell volume of the larvae reared in SW increased gradually during metamorphosis, and

PRL-cell volume of the groups transferred to 1/4 SW increased markedly at all stages examined. An increase in the expression of PRL mRNA has been reported in the flounder larvae during metamorphosis (de Jesus *et al*. 1994), and increases in PRL-cell activity after transfer to low salinity or FW were also observed in the larvae of black sea bream (*Acanthopagrus schlegeli*), amphidromous temperate bass (*Lateolabrax japonicus*), and tilapia (*Oreochromis mossambicus*) (Kimura and Tanaka 1991; Tanaka *et al*. 1996; Ayson *et al*. 1994). The increase in PRL-cell activity may indicate that PRL is involved in the development of low-salinity tolerance during metamorphosis. However, yolk-sac larvae showed better tolerance to 1/8 SW than the larvae 4-14 days after hatching, even though PRL cells were not detected in the pituitary. These results may indicate that PRL is secreted as soon as they are synthesized in the newly-hatched larvae. The other possibility is that the ion and water permeability is extremely low in the yolk-sac larvae, and thus PRL is not required for low-salinity tolerance. The subsequent decrease in tolerance to 1/8 SW may be due to an increase of body surface area caused by mouth opening and gill differentiation of these stages. Gills are the site of active ion secretion in SW and possibly the site of ion uptake in FW (see reviews by Lin and Randall 1995; Flik *et al*. 1995). However, the fish living in FW or a hypotonic environment loses ions and absorbs water mainly through gills because gills occupy the most area of body surface with thin respiratory epithelia. The gills of premetamorphic larvae would be effective in SW adaptation, but may not be enough functional to compensate the ion loss with active ion uptake, or to reduce water permeability in hypotonic environment (1/8 SW). The hyperosmoreguratory ability of flounder gills may develop during metamorphosis.

The whole-body concentration of cortisol in the fish transferred to hypotonic environment was examined by using a newly-developed extraction method. According to Rash *et al*. (1979), lipids interfere with steroid RIAs in two ways. First, lipids form micelles in aqueous solution and

entrap steroids, interfering with the binding between steroids and their antisera. Second, lipids bind to the dextran-coated charcoal, blocking the charcoal absorption of free ligands. By reconstituting and washing with tetrachloromethane, lipids would be removed without forming micelles, and NSB was consequently reduced from 83% to 1%. The extraction with tetrachloromethane was shown to be applicable in other species such as juveniles of yellowtail (*Seriola quinqueradiata*) and red sea bream (*Pagrus major*) (data not shown). No significant difference in whole-body concentration of cortisol was observed between the group transferred to 1/4 SW and the control group. Although it has recently been reported that cortisol is involved in ion uptake in teleosts in FW (see a review by McCormick 1995), the role of cortisol in hyperosmoregulation in flounder larvae and juveniles is still unclear. Elevations of cortisol concentration 12 h after the transfer in both experimental and control groups at all three stages may be due to the transfer stress. Stress-induced increase in whole-body concentration of cortisol has been reported in larval salmonids (Pottinger and Mosuwe 1994; Barry *et al*. 1995a,b), although the peak levels were observed at 1 h poststress. It is also possible that the peak at 12 h reflects diurnal rhythms.

Concentrations of cortisol and thyroid hormones at 0 h of the transfer show basal levels of the hormones during development, so that the differences among three stages reflect ontogenetic changes of the hormones. The highest basal levels of cortisol, T4 and T3 at metamorphic climax are consistent with the previous studies (de Jesus *et al*. 1991; Miwa *et al*. 1988; Tanangonan *et al*. 1989; Tagawa *et al*. 1990).

The whole-body concentrations of thyroid hormones at premetamorphosis and metamorphic climax decreased after low-salinity transfer. Thyroid hormones are well known to induce metamorphosis in flounders (Inui and Miwa 1985; Miwa and Inui 1987), whereas PRL antagonizes the thyroid hormone effects (de Jesus *et al*. 1994). In the present study, PRL-cell

volume increased whereas thyroid hormone concentrations decreased after low-salinity transfer. In striped bass (*Morone saxatilis*), the whole-body concentrations of thyroid hormone increased after the transfer from FW to SW at premetamorphosis and metamorphosis (Parker and Specker 1990). The decreased thyroid hormone concentrations after exposure of the flounder larvae at metamorphic climax may indicate an interaction between thyroid hormones and PRL, although the mode of actions of the hormones is totally unclear. When flounder larvae were exposed to low-salinity water during metamorphosis, metamorphosis is expected to be delayed by increase in PRL and decrease in thyroid hormones. The contribution of endogenous PRL to the control of metamorphosing process would be evaluated experimentally by long-term exposure to low-salinity water.

Chapter 2: Developmental Sequence of Chloride Cells in the Body Skin and Gills of Japanese Flounder (*Paralichthys olivaceus*) Larvae

ABSTRACT—The developmental sequence of chloride cells was examined in both the body skin and gills of Japanese flounder (*Paralichthys olivaceus*) larvae by whole-mount immunocytochemistry using an antiserum specific for Na^+,K^+-ATPase. In premetamorphic larvae at 0 and 4 days after hatching (days 0 and 4), immunoreactive chloride cells were distributed only in the yolk-sac membrane and body skin. Premetamorphic larvae at days 8-18 possessed both cutaneous and branchial chloride cells. Large chloride cells in the skin of premetamorphic larvae often formed multicellular complexes, suggestive of their ion-secreting function. Cutaneous chloride cells decreased in size and density at the beginning of metamorphosis (days 21 and 24), and disappeared at the metamorphic climax (days 28 and 33). In contrast, branchial chloride cells first appeared at day 8, and increased during metamorphosis. These results indicate that the site for ion secretion in seawater may shift from cutaneous to branchial chloride cells during metamorphosis. The appearance of branchial chloride cells before the differentiation of gill lamellae suggests that the primary function of the gills during the early development is ion regulation rather than gas exchanges.

INTRODUCTION

Embryos and larvae of teleosts maintain the internal hydromineral balance, although important osmoregulatory organs in adults such as the gills, kidney and intestine are not fully developed (Alderdice 1988). In juveniles and adults of marine teleosts, chloride cells in the gills are

responsible for the secretion of excess Na^+ and Cl^- in the body fluids (Zadunaisky 1984). In embryos and larvae, chloride cells located in the epithelia covering the yolk and body have been suggested as ion-secreting sites in seawater (Shelbourne 1957; Lasker and Threadgold, 1968; Hwang and Hirano 1985; Alderdice 1988; Hwang 1989, 1990; Ayson *et al*. 1994; Kaneko *et al*. 1995; Shiraishi *et al*. 1997; Sasai *et al*. 1998). Thus, these extrabranchial chloride cells as the major site for ion secretion seem to be taken over by branchial chloride cells as fish grow. However, the spatial shift of chloride cell distribution from the yolk-sac membrane and body skin to gills and their developmental sequence have not been demonstrated through the early development of teleosts.

Marine teleosts generally undergo physiological changes as well as morphological and ecological changes during metamorphosis from larvae to juveniles. Japanese flounder (*Paralichthys olivaceus*) exhibit drastic metamorphosis from pelagic larvae to benthic juveniles, involving the migration of the right eye to the left side of the head. In the present study, to clarify the spatial shift of chloride cell distribution during metamorphosis, we examined the development of both cutaneous and branchial chloride cells in premetamorphic and metamorphic flounder larvae. To detect chloride cells, whole larvae were subjected to immunocytochemical staining with an antiserum specific for Na^+,K^+-ATPase, a key enzyme of ion transport in chloride cells.

MATERIALS AND METHODS

Fish

Naturally spawned eggs of Japanese flounder (*Paralichthys olivaceus*) were collected from a brood-stock tank in the Fisheries Research Station of Kyoto University. Larvae were reared in a polycarbonate tank (500 l) with running seawater. Water temperature was maintained at 18°C, and

the salinity ranged between 30.6 and 32.2 ppt. They were initially fed on rotifers (*Brachionus plicatilis*) cultivated with *Nannochrolopsis* sp. and ω-Yeast (Kyowa Hakko Kogyo, Japan), and later on brine shrimp (*Artemia* spp.) nauplii enriched with Ester-85 (Nippon Chemical Feed, Japan). Samples were taken at nine different developmental stages (Table 1).

Table 1. Relative frequency of chloride cells in the body skin and gills during the early development of Japanese flounder

Days after hatching	Developmental stage[1]	Body length (mm)	Chloride cell frequency[2]	
			skin	gills
premetamorphic larvae				
0		2.5 - 2.6	+	–
4	A	2.8 - 3.1	++	–
8	B	4.0 - 4.3	++	+
14	C	5.0 - 5.5	++	++
18	D	5.8 - 6.1	++	++
metamorphic larvae				
21	E	6.8 - 7.2	+	++
24	F	7.3 - 7.5	±	++
28	G	8.0 - 8.9	–	++
33	H	9.5 - 10.2	–	++

[1]Developmental stages according to Minami (1982).

[2]–, not detected; ±, sparse; +, moderately dense; ++, dense.

The larvae up to 18 days after hatching (day 18) with symmetrical bodies were regarded as premetamorphic larvae, and those on and after day 21 with migrating right eyes as metamorphic larvae. Larvae were anesthetized with MS-222, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 h at 4°C, and preserved in 70% ethanol.

Whole-mount immunocytochemistry

As a specific probe for chloride cells, we used an antiserum specific for Na^+,K^+-ATPase, which is localized in the membrane of the tubular systems distributed extensively over the

cytoplasm of chloride cells (Karnaky *et al.* 1976; Hootman and Philpott 1979). The antiserum was raised in a rabbit against a synthetic peptide corresponding to part of the highly conserved region of the Na^+,K^+-ATPase α-subunit (Ura *et al.* 1996). It has been well documented that the anti-Na^+,K^+-ATPase specifically detects both cutaneous and branchial chloride cells in several teleosts (Ura *et al.* 1996; Uchida *et al.* 1996; Shiraishi *et al.* 1997; Sasai *et al.* 1998, 1999).

Whole-mount immunocytochemistry based on the avidin-biotin-peroxidase complex (ABC) method (Hsu *et al.* 1981) was carried out following the method of Ohtani *et al.* (1989) using commercial reagents (Vectastain Elite ABC kit, Vector Laboratories, USA). The right operculum of larvae after day 4 was removed prior to the immunostaining in order to enhance penetration of solutions to the gills. After treatment with 0.1% sodium cyanoborohydride in 0.01 M phosphate-buffered saline (PBS, pH 7.2) for 1 h, the samples were incubated sequentially with: 1) rabbit anti-Na^+,K^+-ATPase diluted 1:500 for 20 h at 4°C, 2) biotinylated goat anti-rabbit IgG for 20 h at 4°C, 3) ABC reagent for 20 h at 4°C, and 4) 0.03% 4-Cl-1-naphthol in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.003% hydrogen peroxide for 20 min. The antisera and ABC reagent were diluted with PBS containing 0.05% Triton X-100, 10% normal goat serum, 0.1% bovine serum albumin and 0.01% sodium azide. The whole sample and separated gill arches were mounted on a slide with glycerin.

The image of the body surface on the left side was digitized with a CCD video camera (Victor, Japan) and an image processor (ARGUS 20, Hamamatsu photonics, Japan), and the size and density of immunopositive chloride cells in the body skin were measured on an Apple Macintosh computer using the public domain NIH Image program (available on the Internet at http://rsb.info.nih.gov/nih-image/). The quantitative analysis was made on cutaneous chloride cells only in the yolk-sac membrane and the skin of trunk and tail, in which chloride cells were uniformly

distributed during early developmental stages. The head region, finfold of premetamorphic larvae and fins of metamorphic larvae were excluded from the measurement, because the sparseness of chloride cells in these regions was considered inappropriate for density measurement. The quantitative analysis of branchial chloride cells was not carry out, since it was difficult to measure the precise cell density and size in the complicated, three-dimensional structures of the gills.

RESULTS

Chloride cells in the skin

In newly-hatched flounder larvae (day 0), weakly-immunopositive but large chloride cells were detected in the epithelia covering both the yolk and body (Figs. 1A, B). In premetamorphic larvae at days 4-18, a large number of immunopositive chloride cells were distributed in the body skin (Figs. 1C-F). Chloride cells in the skin of newly-hatched and premetamorphic larvae were characterized by the formation of cell clusters: several chloride cells congregated to form a multicellular complex as indicated by the presence of more than one immunonegative nuclei (Figs. 1B, D, F). Especially, large chloride cell complexes were observed in the abdominal region. In metamorphic larvae at day 21 just starting the right-eye migration, both size and density of chloride cells decreased (Figs. 1G, H). In contrast with premetamorphic larvae, these chloride cells were present individually and did not form multicellular complexes. No chloride cells were detected in the body skin of metamorphic-climax larvae at days 28 and 33. However, small immunopositive cells not forming cellular complexes were found in the pectoral, dorsal and anal fins of metamorphic larvae at days 21-33, although few chloride cells were detectable in the pectoral fin and finfold of premetamorphic larvae at days 0-18.

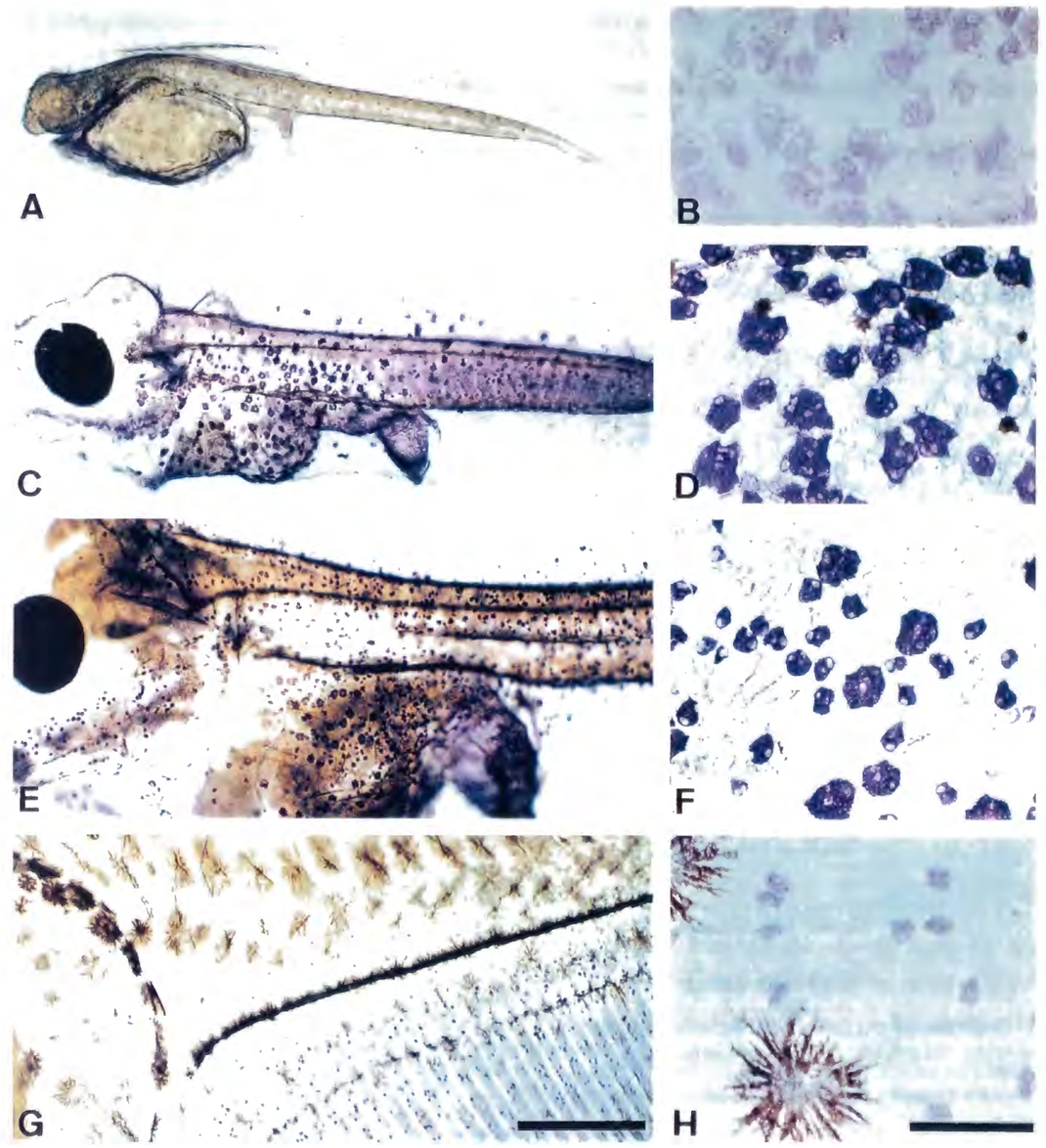


Fig. 1. Cutaneous chloride cells of flounder larvae at day 0 (**A**, **B**), day 8 (**C**, **D**), day 18 (**E**, **F**) and day 24 (**G**, **H**), detected by whole-mount immunocytochemistry using an antiserum specific for Na^+,K^+-ATPase. (**B**, **D**, **F**, **H**) Magnified views of separated yolk-sac membrane or body skin in the abdominal region. Bars: (**A**, **C**, **E**, **G**) 500 μm; (**B**, **D**, **F**, **H**) 100 μm.

Figure 2 shows developmental changes in size-frequency distributions of chloride cells and chloride cell complexes in the yolk-sac membrane and body skin.

During the early development, the mean size of chloride cells decreased concomitant with the disappearance of chloride cell complexes. The density of chloride cells was decreased drastically at the beginning of metamorphosis (days 21 and 24), and the cells disappeared at the metamorphic climax at days 28 and 33 (data not shown).

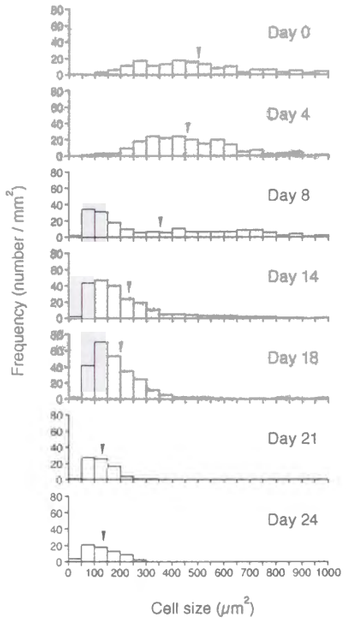


Fig. 2. Developmental changes in size-frequency distributions of chloride cells in the yolk-sac membrane and body skin of flounder larvae. Data obtained from 4 individuals were combined at each day. Arrows indicate the mean values. No chloride cell is detectable in the body skin at days 28 and 33.

Chloride cells in the gills

The gills were not distinguishable in the whole-mount preparation of newly-hatched larvae at day 0. Four pairs of gill arches were recognizable in larvae at day 4, although the gill filament and lamella were not yet differentiated. Chloride cells were not yet detected in the gill arches at this early developmental stage. In premetamorphic larvae at day 8, gill filaments sprouted from the gill arches but the lamella was not differentiated. Immunoreactive chloride cells were observed in the filament epithelia of all the four pairs of gills (Fig. 3A). In premetamorphic larvae at day 18, the filaments were further developed and the lamellae were extended from the filaments. Chloride cells were extensively distributed in the filaments, but were absent in the lamellae (Fig. 3B). In metamorphic larvae at day 24, when the filaments and lamellae were extended further, chloride cells were overspread in the filaments, whereas no chloride cell was observed in the lamellae (Fig. 3C).

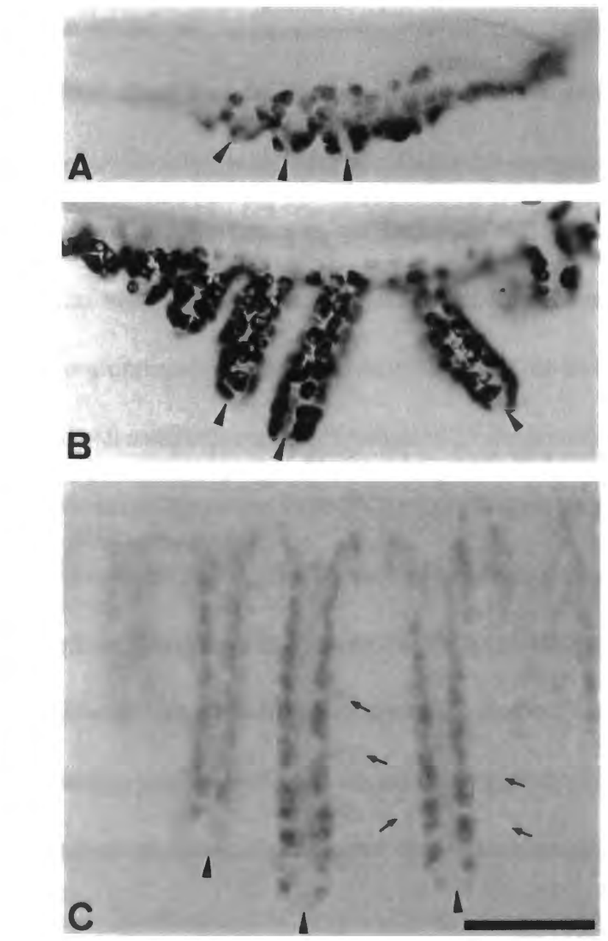


Fig. 3. Branchial chloride cells in the first gill arch of the left side of flounder larvae at day 8 (**A**), day 18 (**B**) and day 24 (**C**), detected by whole-mount immunocytochemistry using an antiserum specific for Na^+,K^+-ATPase. The gill lamellae are already differentiated at day 18 (**B**), although they are not visible because of being out of focus. Arrowheads and arrows indicate gill filaments and lamellae, respectively. Bar: 100 μm.

DISCUSSION

In the present study, the developmental sequence of both cutaneous and branchial chloride cells was clarified in the early life stages of Japanese flounder by the whole-mount immunocytochemistry. As summarized in Table 1, the chloride cell distribution shifts from the body skin to the gills during the early development. Such a spatial shift of chloride cell distribution is closely associated with metamorphosis.

Larvae at days 0 and 4 possessed only cutaneous chloride cells, which seem to be the only functional site for ion secretion at these early developmental stages. In premetamorphic larvae at days 8-18, a large number of chloride cells were observed in both the body skin and gills. Although the mean size of cutaneous chloride cells decreased gradually during the development, the cell density increased and reached the highest value at day 18, just before the beginning of metamorphosis. We did not carry out a quantitative analysis of branchial chloride cells, since it was difficult to measure the precise cell density and size in the complicated, three-dimensional structures of the gills. However, the branchial chloride cell number certainly increased during the development. These findings suggest that both cutaneous and branchial chloride cells function cooperatively as the site for ion secretion at these premetamorphic stages. Later on, cutaneous chloride cells disappeared and branchial chloride cell number increased further during metamorphosis. These observations clearly indicate that the site for ion secretion shifts from cutaneous to branchial chloride cells during flounder metamorphosis.

During the metamorphosis of Japanese flounder, a series of physiological changes occurs: larval types of the digestive system, muscles and erythrocytes change into adult types (Tanaka 1973; Yamano *et al*. 1991; Miwa and Inui 1991; Miwa *et al*. 1992). The shift from cutaneous to

branchial chloride cells would be categorized as one of such physiological changes during metamorphosis. Besides Japanese flounder, many other marine teleosts also exhibit metamorphosis to varying degrees. The shift from cutaneous to branchial chloride cells observed in Japanese flounder could be expected to occur during metamorphosis in those teleosts.

A large proportion of cutaneous chloride cells in premetamorphic flounder larvae formed multicellular complexes. Similar chloride cell complexes have been reported in the yolk-sac membrane and body skin of tilapia (*Oreochromis mossambicus*) and Japanese eel (*Anguilla japonica*) embryos and larvae reared in seawater (Shiraishi *et al*. 1997; Sasai *et al*. 1998). Shiraishi *et al*. (1997) have proved that a chloride cell complex in the yolk-sac membrane of seawater-adapted tilapia larvae consists of a main chloride cell and adjacent accessory cells, which interdigitate with each other to form multiple junctions. The complex is considered to be advantageous to Na^+ secretion, since Na^+ secretion may occur down its electrochemical gradient via a paracellular pathway in the complex (Marshall 1995; McCormick 1995). Therefore, the occurrence of chloride cell complexes in the body skin of premetamorphic flounder larvae would provide morphological evidence that those cells function as ion-secreting sites in seawater.

It should be noted that branchial chloride cells first appeared on the gill filaments before the differentiation of lamellae at day 8. Since the lamellae are largely involved in gas exchanges by enlarging the branchial surface area, the primary function of the gills during the early development seems to be ion regulation rather than gas exchanges.

In metamorphic larvae, although the gills were equipped with filaments and lamellae, chloride cells were distributed only in the filaments but not in the lamellae. Two distinct types of chloride cells have been reported in the gill filaments and lamellae of chum salmon (*Oncorhynchus keta*) fry and adults and Japanese eel adults (Uchida *et al*. 1996, 1997; Sasai *et al*. 1999). In the gills of these

species adapted to fresh water, chloride cells were found in both filaments and lamellae. After transfer to seawater, the number of lamellar chloride cells decreased and filament chloride cells were activated. These morphological observations suggest that filament and lamellar chloride cells are involved in seawater and freshwater adaptation, respectively. Therefore, filament chloride cells of flounder larvae may be important in seawater adaptation, most probably acting as the site for salt secretion in hyperosmotic environments. In nature, flounder larvae migrate from offshore areas to estuaries during metamorphosis, when the low-salinity tolerance develops to some extent (see Chapter 1). Observations on branchial chloride cell alteration during the adaptation to hypoosmotic environments would be of considerable interest to explore diverse functions of chloride cells in iono- and osmoregulation.

Chapter 3: *In vivo* Sequential Changes in Chloride Cell Morphology in the Yolk-Sac Membrane of Mozambique Tilapia (*Oreochromis mossambicus*) Embryos and Larvae during Seawater Adaptation

ABSTRACT—*In vivo* sequential changes in chloride cell morphology were examined in the yolk-sac membrane of Mozambique tilapia (*Oreochromis mossambicus*) embryos and larvae transferred from fresh water (FW) to seawater (SW). Labeling chloride cells with DASPEI, a fluorescent probe specific for mitochondria, we observed sequential changes in individual chloride cells by confocal laser scanning microscopy. In fish transferred from FW to SW at 3 days after fertilization, 79% of chloride cells survived at 96 h after transfer, and each cell showed a remarkable increase in size. In contrast, the cell size did not change greatly in fish kept in FW. The numbers of both disappearing and newly-appearing cells in FW fish were significantly larger than in SW fish, suggesting a higher turnover rate in FW than in SW. Using whole-mount immunocytochemistry with anti-Na^+, K^+-ATPase and differential interference contrast (DIC) optics, we classified chloride cells in the fixed samples into three developmental stages: a single chloride cell without an apical pit, a single chloride cell with an apical pit, and a multicellular complex of chloride cells with a common apical pit. Immunofluorescence and DIC observations also revealed that single chloride cells were enlarged and frequently dented by newly-differentiated accessory cells to form multicellular complexes during SW adaptation. These results indicate that FW-type, single chloride cells are transformed into SW-type, chloride cell complexes during SW adaptation, suggesting the excellent plasticity in altering ion-transporting functions of chloride cells in the yolk-sac membrane of tilapia embryos and larvae.

INTRODUCTION

In adult teleosts, chloride cells in the gill epithelium are the major site for ionic regulation. Chloride cells are characterized by numerous mitochondria and an extensive tubular system, in which ion-transporting enzymes such as Na^+,K^+-ATPase are located (Karnaky *et al.* 1976; Philpott 1980; McCormick 1995). These cells are involved in the secretion of excess ions in the body fluid in seawater (SW), and possibly in ion uptake in fresh water (FW) (Foskett and Scheffey 1982; Zadunaisky 1984; Avella and Bornancin 1990; Marshall 1995; Perry 1997). In embryos and larvae of several teleosts, chloride cells have been detected in the epithelia covering the yolk and body, and these extrabranchial chloride cells are considered to be the site for ionic regulation during early developmental stages without functional gills (Shelbourne 1957; Lasker and Threadgold, 1968; Hwang and Hirano 1985; Alderdice 1988; Hwang 1989, 1990; Ayson *et al.* 1994; Hwang *et al.* 1994; Kaneko *et al.* 1995; Shiraishi *et al.* 1997; Sasai *et al.* 1998a; Hiroi *et al.* 1998a).

Mozambique tilapia (*Oreochromis mossambicus*) can mature and breed in both FW and SW, and the embryos can survive direct transfer from FW to SW and *vice versa*. Ayson *et al.* (1994) reported that tilapia embryos and larvae adapted to both FW and SW possess numerous chloride cells in the yolk-sac membrane, and that chloride cells in SW embryos and larvae are larger than those in FW. Moreover, chloride cells in the yolk-sac membrane become larger in response to transfer from FW to SW, whereas the chloride cell size becomes smaller when transferred from SW to FW. According to Shiraishi *et al.* (1997), well-developed chloride cells in SW tilapia embryos and larvae form multicellular complexes, consisting of chloride cells and accessory cells. These morphological observations suggest the occurrence of FW- and SW-type chloride cells in the yolk-sac membrane of tilapia embryos and larvae, as is the case with gill chloride cells in adult fish (Pisam and Rambourg 1991; Uchida *et al.* 1996; Sasai *et al.* 1998b). The strong euryhalinity of tilapia embryos and larvae seem to be attributed to these chloride cells, which may function as the sites for ion uptake and secretion in FW and SW, respectively. However, the mechanism for altering their functions when exposed to different osmotic environments has not yet documented.

From a physiological and morphological point of view, it is of great interest to examine whether chloride cells are replaced with newly-differentiated cells of a different function when transferred from FW to SW or from SW to FW, or the same chloride cells function in both FW and SW. The most effective way to answer this question would be to examine sequential changes in individual chloride cells during adaptation to a different salinity. However, conventional methods do not allow us to follow time-course changes in the same chloride cells. Separated gill filaments of adults are not suitable materials to examine the time-course changes of chloride cells, because of their complex, three-dimensional structures. Primary cultures of the gill epithelium may also be ineffective, since dispersed chloride cells do not survive under *in vitro* conditions (Pärt and Bergström 1995).

In the present study, we carried out an *in vivo* follow-up observation of individual chloride cells in the yolk-sac membrane of tilapia embryo and larvae transferred from FW to SW. An intact fish were immersed in a medium containing a mitochondrial fluorescent probe, which has been used to identify chloride cells, and morphological changes in individual chloride cells were examined continuously under a confocal laser scanning microscope. Meanwhile, chloride cells in the fixed yolk-sac membrane were examined by immunocytochemistry with an antiserum specific for Na^+,K^+-ATPase, a key enzyme for ion transport in chloride cells, in order to support the results of the *in vivo* experiment and to observe more detailed structures of these cells at a higher magnification.

MATERIALS AND METHODS

Fish

Mature tilapia (*Oreochromis mossambicus*) were maintained in tanks with recirculating FW (Na^+, 0.74 mM; Ca^{2+}, 0.54 mM; Mg^{2+}, 0.26 mM) at 25°C. Fertilized eggs were obtained from the mouth of brooding females at 3 days after fertilization. Since the chorion of embryos is not permeable to a fluorescent dye and dissolved at hatching to cause water fouling, the chorion was torn off with sharp-pointed forceps under a dissecting microscope. In the following experiment, water temperature was maintained at 25°C. Tilapia embryos typically hatch after 5-day incubation at 25°C, and yolk absorption is completed by 10 days after hatching. Larvae were not fed during the experiment.

Vital staining and sequential observation of individual chloride cells

Dechorionated embryos (3 days after fertilization or 2 days before hatching) were incubated in FW containing 250 μM 2-(4-dimethylaminostyryl)-1-ethylpyridinium iodide (DASPEI, Sigma, St. Louis, Mo.) for 6 h prior to an initial observation. DASPEI is a mitochondrial vital probe which has been used to identify chloride cells (Bereiter-Hahn 1976; Karnaky *et al.* 1984). An individual embryo was then briefly rinsed in DASPEI-free FW, and placed on a chamber filled with the ambient water using a large-mouthed pipette. The chamber consisted of a coverslip (24x24 mm) and a spacer, which was adjusted slightly thinner than the thickness of the yolk sac. The chamber was then put on a glass slide and covered with another coverslip, so that the fish was sandwiched between two coverslips. The flattened surface at the top of the yolk sac was examined with a Zeiss 310 confocal laser scanning microscope (LSM, Carl Zeiss, Oberkohen, Germany). It is practically

difficult to observe DASPEI-positive chloride cells in the yolk-sac membrane using a conventional fluorescence microscope because of strong autofluorescence of the yolk. In contrast, LSM enables us to observe the sectional image of the yolk-sac membrane, which is not affected by the autofluorescence of the yolk. The 488 nm line of an argon ion laser was used as the excitation wavelength, and the emission was collected at 515-565 nm. An area of the yolk-sac membrane where chloride cells were densely distributed was selected, and a confocal image of 0.41 mm^2 was obtained with a 20x/0.50 objective lens.

After the initial observation, the coverslip on the top was carefully removed, and the chamber including the embryo was immersed in 10 ml artificial SW (Na^+, 490 mM; Ca^{2+}, 16 mM; Mg^{2+}, 66 mM; Jamarin Laboratory, Osaka, Japan) containing 250 μM DASPEI. At 2, 6 and 12 h after transfer to SW containing DASPEI, confocal images were obtained from the area including most of chloride cells identified in the initial observation. The distribution pattern of melanophores in the yolk sac was available for recognizing the same area. After that, confocal images of the same area were obtained repeatedly at 6-h intervals until 96 h after transfer to SW. The incubation medium (DASPEI-containing SW) was replaced with DASPEI-free SW at 6-h intervals: the fish was incubated in DASPEI-free SW for 12-18, 24-30, 36-42, 48-54, 60-66, 72-78 and 84-90 h, and in SW containing DASPEI for 18-24, 30-36, 42-48, 54-60, 66-72, 78-84 and 90-96 h after SW transfer. As a control, the other embryos were kept in FW and examined in the same procedure for SW-transferred embryos. The experiment was conducted with two broods produced by different parents.

The successive confocal images obtained from both FW and SW embryos and larvae at 0, 24, 48, 72 and 96 h after transfer were used for morphometrical analyses of chloride cells. The size of DASPEI-positive chloride cells were measured on an Apple Macintosh computer using the public

domain NIH Image program (available on the Internet at http://rsb.info.nih.gov/nih-image/).

Immunofluorescence and DIC observations

Another brood of dechorionated embryos at 3 days after fertilization were separated into two groups: half of the embryos were transferred directly to artificial SW, and the other half were maintained in FW. The ambient water was renewed once a day. The FW embryos at 3 days after fertilization (2 days before hatching), and the larvae of both groups at 48 and 96 h after the transfer (corresponding to 0 and 2 days after hatching, respectively) were anesthetized with 2-phenoxyethanol, and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 50 min at 4°C. The yolk sac was then incised, the yolk was carefully scraped out, and the connective tissue and capillaries under the basal membrane were removed with sharp-pointed forceps. The embryos and larvae were further fixed overnight at 4°C and preserved in 70% ethanol.

Whole-mount immunocytochemistry was carried out following the method of Ohtani *et al.* (1989) with some modifications. After treatment with 0.1% sodium cyanoborohydride in 0.01 M phosphate-buffered saline (PBS, pH 7.2) for 1 h, the samples were incubated overnight at 4°C with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-Na^+, K^+-ATPase diluted 1:500 with PBS containing 0.05% Triton X-100, 10% normal goat serum, 0.1% bovine serum albumin, 0.02% keyhole limpet hemocyanin and 0.01% sodium azide. After rinse in PBS for 1 h, the yolk-sac membrane was removed from the body trunk, mounted on a slide with PBS and examined by LSM. The excitation and emission wavelengths were the same as the DASPEI observation.

Three confocal images corresponding to 1.23 mm^2 were obtained from each sample with a 20x/0.50 objective lens, and the size and density of immunopositive chloride cells were measured by using the NIH Image program. For more detailed observations on chloride cell structures, both

confocal fluorescence images and differential interference contrast (DIC) images were also taken with a 63x/1.4 oil-immersion objective lens. For size and density measurement, two or more immunopositive chloride cells which shared one apical pit and formed a multicellular complex were regarded as one cell; immunopositive chloride cells which touched but possessed their own apical pits were measured separately.

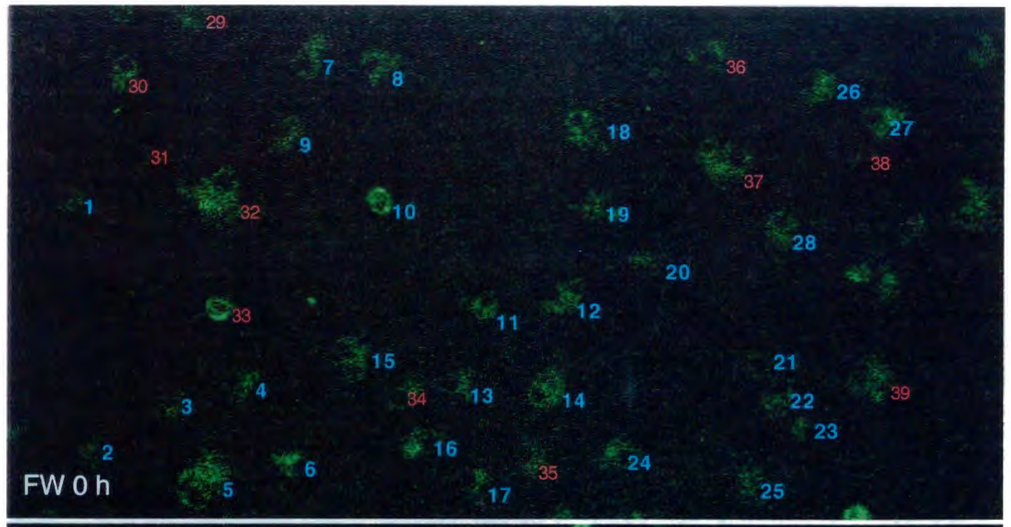
Antibodies

A polyclonal antibody was raised in a rabbit against a synthetic peptide corresponding to part of the highly conserved region of the Na^+, K^+-ATPase α-subunit (Ura *et al.* 1996). The amino acid sequence of the synthetic peptide was Val-Thr-Gly-Val-Glu-Glu-Gly-Arg-Leu-Ile-Phe-Asp-Asn-Leu-Lys-Lys-Cys. The antibody was further purified by affinity chromatography and conjugated to FITC. The specificity of the antibody was confirmed by western blotting, and the details will be described elsewhere.

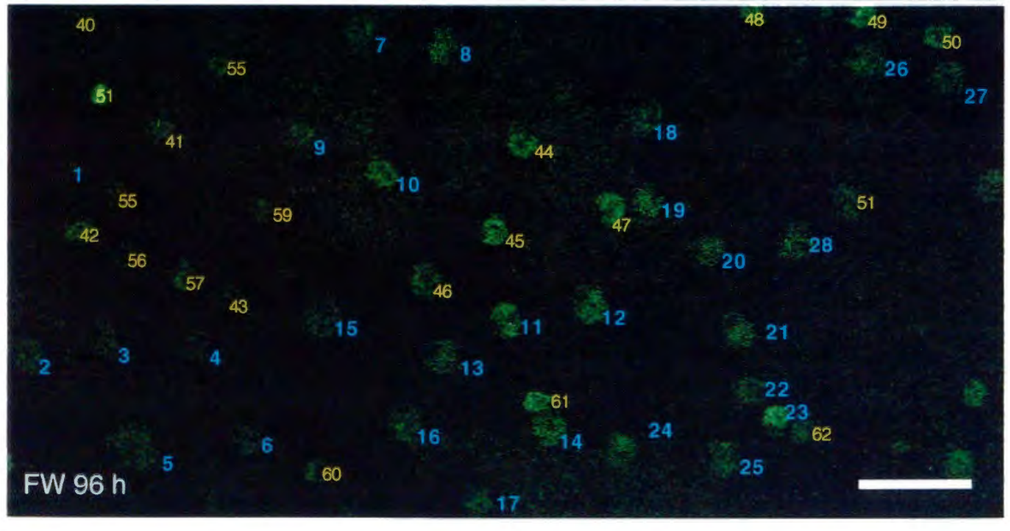
RESULTS

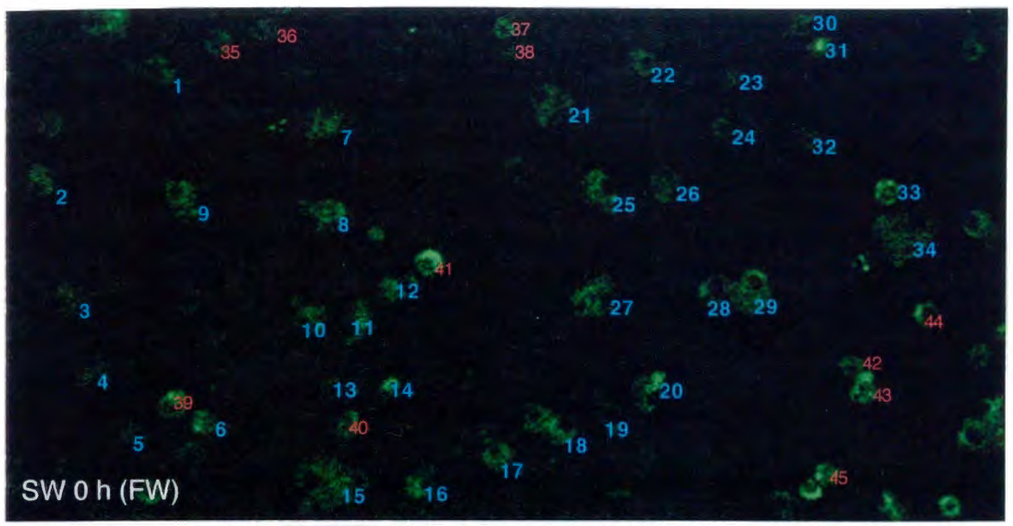
Vital staining and sequential observation of individual chloride cells

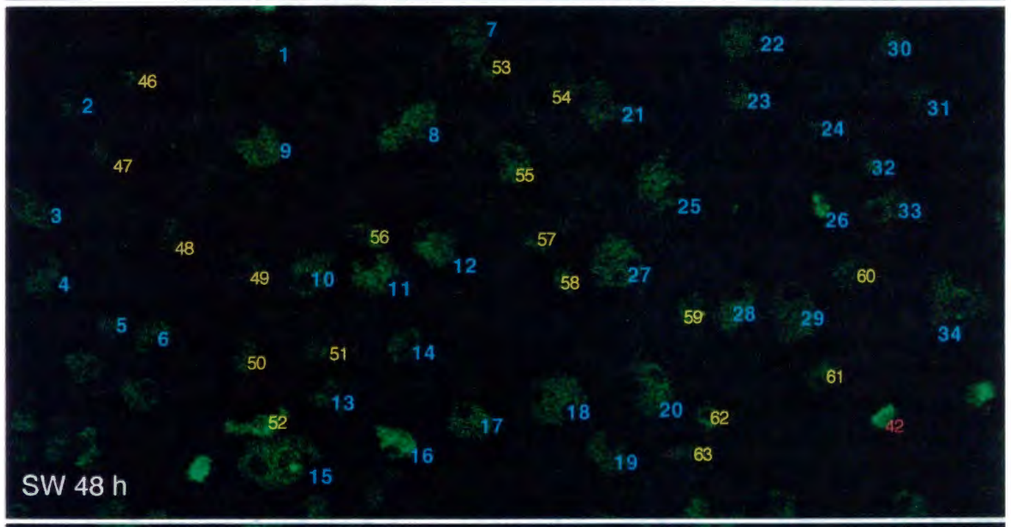
During the experimental period for 96 h, sequential images of DASPEI-positive chloride cells in the yolk-sac membrane were successfully obtained from three individuals kept in FW (one and two individuals from two different broods), and from four individuals transferred to SW (two individuals each from two broods). The representative confocal images of FW and SW individuals at 0, 48 and 96 h are shown in Figure 1.


FW 0 h


FW 48 h


FW 96 h


SW 0 h (FW)


SW 48 h

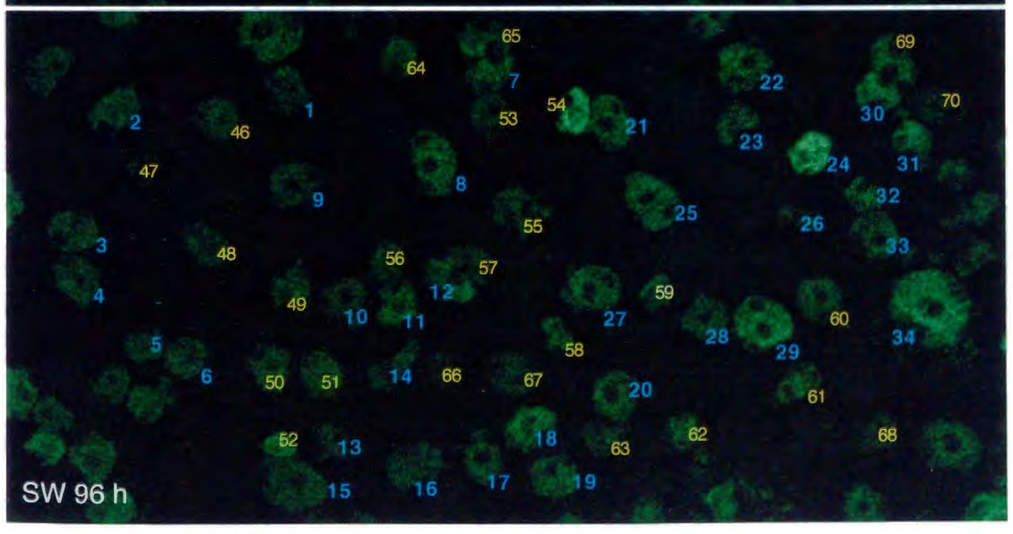

SW 96 h

Fig. 1. Sequential confocal images of DASPEI-stained chloride cells in the yolk-sac membrane of an individual kept in FW (**FW 0 h, FW 48 h, FW 96 h**) and that transferred from FW to SW (**SW 0 h, SW 48 h, SW 96 h**) at 0 h, 48 h and 96 h. Chloride cells detectable throughout the experiment are labeled with blue numbers. Cells existing at 0 h and disappearing thereafter are labeled with red; those newly appearing but disappearing with pink; and newly appearing and surviving until 96 h with yellow. Bar: 50 μm.

The distribution patterns of chloride cells were largely maintained during the experiment, unless the yolk-sac membrane was not damaged. Consequently, each chloride cell was able to be readily identified by the sequential observations at 6-h intervals.

A time-course change in the size of each chloride cell identified in Figure 1 is illustrated in Figures 2A and B. In both FW fish and those transferred to SW, a large proportion of chloride cells were detectable throughout the experiment, whereas the other cells appeared or disappeared during this period. Most chloride cells tended to increase in size after SW transfer, while the cell size did not change greatly in FW. This is further confirmed by changes in average size of chloride cells obtained from three FW and four SW individuals (Figs 2C and D). Based on their patterns of appearance, chloride cells which survived until 96 h were classified into five subgroups: cells pre-existing at 0 h, and those appearing at 24 h, 48 h, 72 h and 96 h. Figures 2C and D also show changes in average sizes of those subgroups in FW and SW. In FW individuals, the size of pre-existing cells remained constant throughout the experiment. The sizes of cells appearing at 24 h, 48 h and 72 h also remained constant, although these cells were somewhat smaller than the pre-existing cells at the time of their appearance. In contrast, the pre-existing cells showed a remarkable increase in size after SW transfer. Although the cells appearing after SW transfer were comparable to those in FW at the beginning, their size increased thereafter.

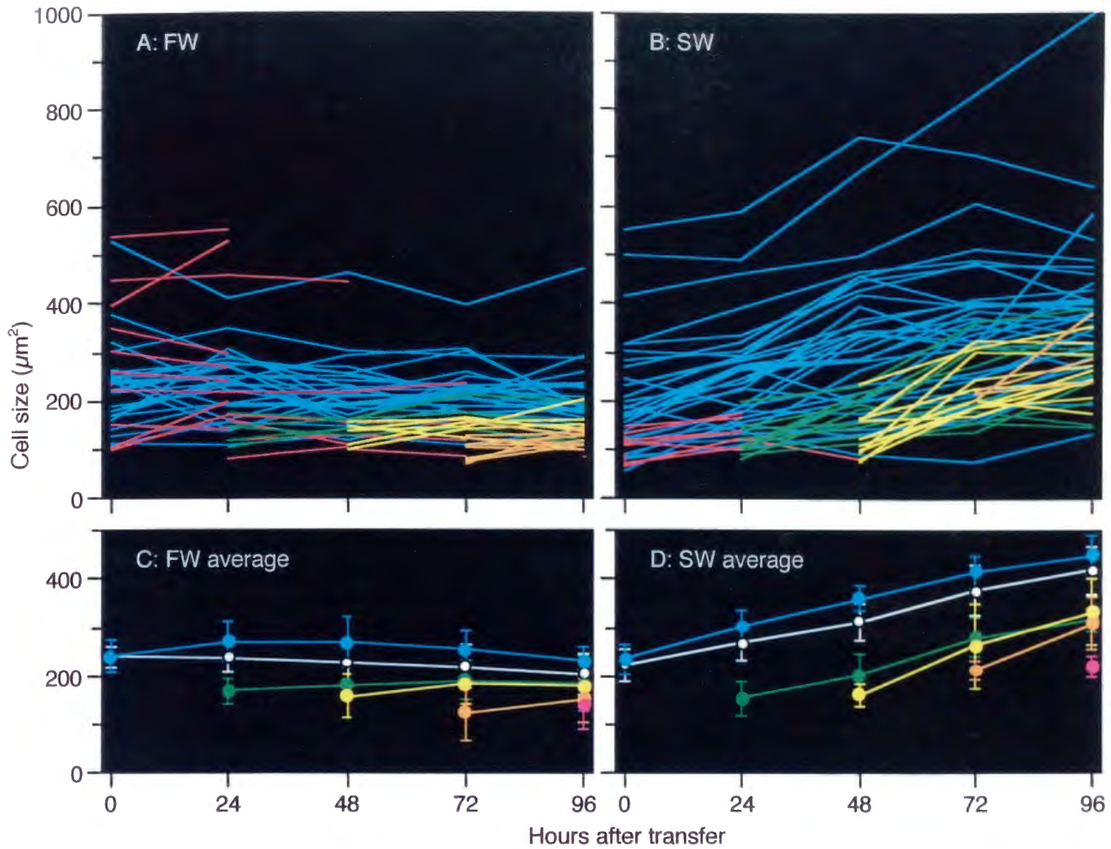


Fig. 2. Time-course changes in the size of each chloride cells in the yolk-sac membrane of the FW and SW individuals shown in Figure 1 (**A, B**) and the average size of chloride cells obtained from three FW and four SW individuals (**C, D**). Chloride cells detectable throughout the experiment are plotted in blue. Cells appearing at 24 h, 48 h, 72 h and survive until 96 h are shown in green, yellow and orange, respectively, while those appearing at 96 h in magenta. Disappearing cells are shown in red. The average size of all chloride cells are plotted in white in **B** and **D**. Vertical bars represent standard deviations.

Time-course changes in the number of chloride cells are shown in Figure 3. In FW individuals, the number of pre-existing chloride cells was decreased at 48 h, and 67 ± 3% (mean ± S. D.) of the cells remained at 96 h, while new cells appeared constantly at 24, 48 and 72 h. In SW individuals, the number of pre-existing cells decreased gradually at 24 and 48 h, kept a constant level at 48-96 h, and 79 ± 5% of the cells remained at 96 h. A considerable number of cells appeared at

24 and 48 h, and most of them remained until 96 h. The number of pre-existing cells which remained at 96 h in FW individuals was significantly smaller than that in SW individuals (Student's t test, $P < 0.05$). The total number of both disappearing and newly-appearing cells during 96 h in FW individuals was significantly larger than in SW individuals ($P < 0.05$).

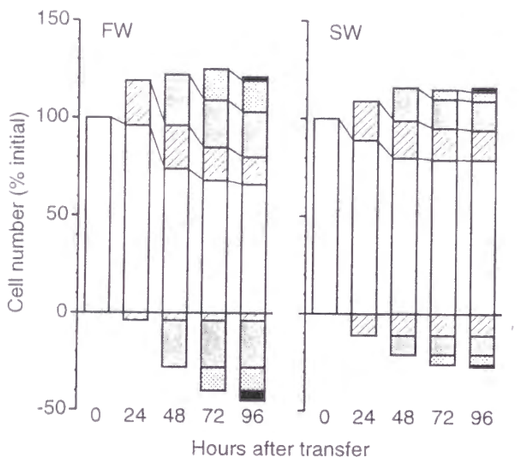


Fig. 3. Time-course changes in the number of chloride cells in the yolk-sac membrane of FW and SW individuals. Data obtained from three FW and four SW individuals were averaged at each observation time. The number of newly-appearing cells is piled up, and that of disappearing cells is shown negatively.

Immunofluorescence and DIC observations

Immunofluorescence observations on chloride cells in the yolk-sac membrane of the fixed samples also revealed changes in their morphology in FW and SW-transferred fish. Size-frequency distributions of immunopositive chloride cells of FW and SW individuals at 0, 48 and 96 h are shown in Figure 4. In FW, chloride cells were consistently small, the mean size being 200-250 μm^2. Following transfer to SW, chloride cells became larger, and the mean size reached about 400 μm^2 at 96 h.

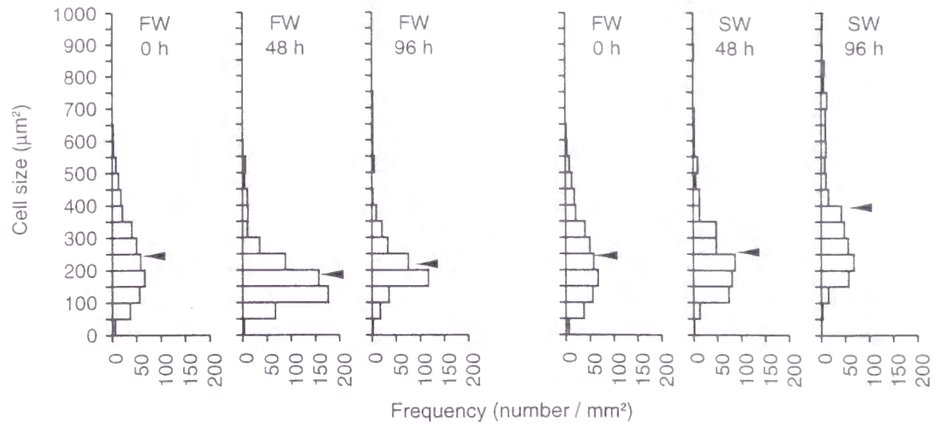


Fig. 4. Time-course changes in size-frequency distributions of Na^+, K^+-ATPase immunopositive chloride cells in the yolk-sac membrane of fixed samples. Data obtained from four FW and four SW individuals are averaged at each sampling time. Arrows indicate the mean values.

DIC observations revealed three distinct cell types in the yolk-sac membrane; that is, pavement, chloride and putative undifferentiated cells (Fig. 5). Pavement cells with ridge structures and apical openings of chloride cells were clearly observed on the outer surface of the yolk-sac membrane (Figs. 5A, D, G), and underlying chloride cells and putative undifferentiated cells were detectable on a deeper plane (Figs. 5B, E, H). Both chloride and undifferentiated cells possessed a nucleus of a similar smooth appearance; the cytoplasm of chloride cells was readily distinguishable by their rough appearance, while that of undifferentiated cells was indistinct. Undifferentiated cells were abundantly distributed all over the yolk-sac membrane, being frequently attached to chloride cells. Na^+, K^+-ATPase immunoreactivity was detected only in the cytoplasm of chloride cells (Figs. 5C, F, I).

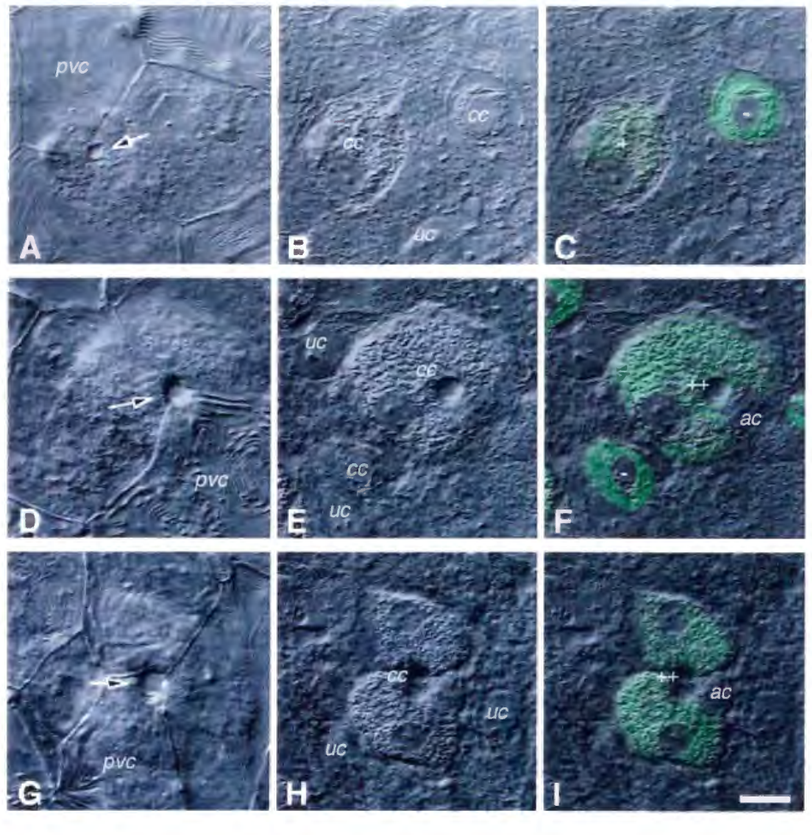


Fig. 5. DIC and immunofluorescence images of chloride cells in the yolk-sac membrane of fixed samples. **A-C** FW fish at 96 h; **D-I** SW fish at 96 h. The ridge structures and boundaries of pavement cells (*pvc*) and apical pits (*arrows*) of chloride cells are observable on the surface (**A, D, G**), while the underlying chloride cells (*cc*)and undifferentiated cells (*uc*) are in focus on a deeper plane of the same tissues (**B, E, H**). Na^+, K^+-ATPase-immunopositive chloride cells are classified into three subtypes: a single chloride cell without an apical pit (-); a single chloride cell with an apical pit open (+); and a multicellular complex of chloride cells and accessory cells (*ac*) with a common apical pit (++) (**C, F, I**). Bar: 10 μm.

The combination of DIC and immunofluorescence images allowed us to classify immunopositive chloride cells into three subtypes: a single chloride cell without an apical pit; a single chloride cell with an apical pit open; a multicellular complex of chloride cells with a common apical pit (Fig. 5). Although single chloride cells without a pit were small, the cytoplasm showed strong immunoreactivity to Na^+, K^+-ATPase, and an immunonegative nuclei was located in the

center (Figs. 5A, B, C). Single chloride cells with a pit possessed larger cytoplasm, the most part of which was immunopositive; the nuclei were located peripherally (Figs. 5A, B, C). The multicellular complex of chloride cells usually consisted of an immunopositive main chloride cell and one or more accessory cells (Figs. 5D, E, F), and occasionally consisted of two or more immunopositive chloride cells of similar size, being also accompanied by accessory cells (Fig. 5G, H, I). All multicellular complexes possessed an apical pit. The cytoplasm of accessory cells was slightly immunopositive or immunonegative to Na^+, K^+-ATPase. Accessory cells dented main chloride cells to a variable extent (Fig. 6). The main chloride cells frequently enveloped accessory cells, so that the boundary between these cells was indistinct (Fig. 6D).

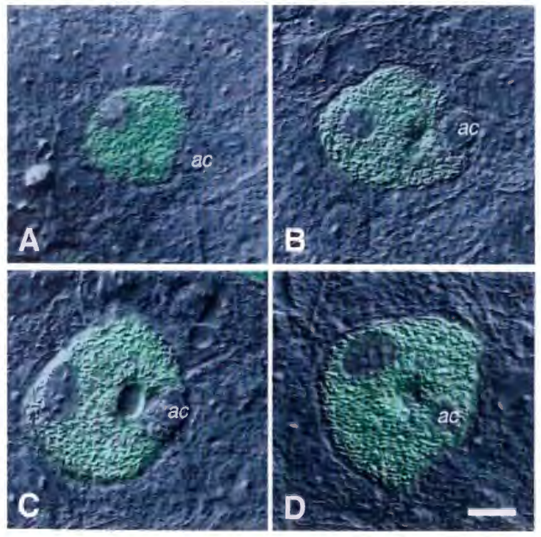


Fig. 6. A series of DIC and immunofluorescence images of multicellular complexes of chloride cells, arranged in the order of presumptive developmental stages. At first, an accessory cell (*ac*) is attached to a single, small chloride cell (**A**). The chloride cell becomes larger and dented by the accessory cell (**B, C**). The accessory cell is finally enveloped by the chloride cell, so that the boundary between these cells is indistinct (**D**). Bar: 10 μm.

Although all three subtypes of chloride cells were found in both FW and SW individuals throughout the experiment, the numbers of those chloride cell subtypes varied greatly in FW and SW (Fig. 7). In FW individuals, the numbers of single chloride cells both with and without a pit was increased at 48 and 96 h, whereas chloride cell complexes decreased during the experiment. In SW individuals, single chloride cells also increased at 48 h but decreased at 96 h. In contrast with FW

experiment.

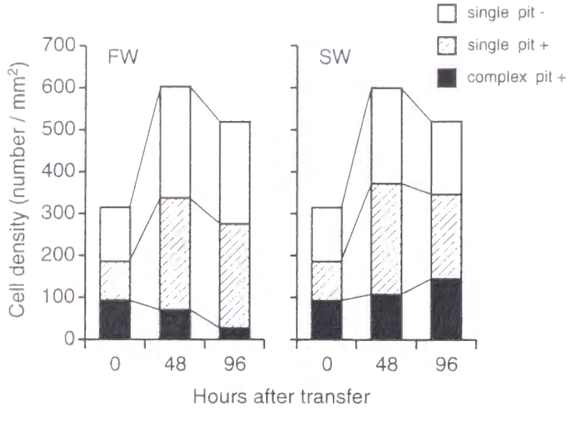


Fig. 7. Time-course changes in the density of three types of Na^+, K^+-ATPase-immunopositive chloride cells in the yolk-sac membrane of the fish maintained in FW and those transferred to SW. Chloride cells are classified into a single cell without a pit, a single cell with a pit and a multicellular complex with a common pit. Data obtained from four FW and four SW individuals are averaged at each sampling time.

DISCUSSION

In the present study, observing DASPEI-positive chloride cells in the yolk-sac membrane of tilapia embryos and larvae *in vivo*, we demonstrated for the first time the sequential changes in individual chloride cell morphology during SW adaptation. In addition to the *in vivo* sequential observations on DASPEI-stained chloride cells, we examined chloride cells in the fixed yolk-sac membrane by whole-mount immunocytochemistry with anti-Na^+, K^+-ATPase. The time-course changes in average size and number of chloride cells after transfer to SW examined by the *in vivo* observations accord well with those obtained from whole-mount immunocytochemistry in the fixed tissue. Thus, the treatment of the live fish with DASPEI is considered to have little influence on the chloride cell response.

In the fish transferred from FW to SW, individual chloride cells increased their size. The present observation on the fixed sample at a higher magnification and our previous studies (Shiraishi *et al.* 1997) indicate that chloride and accessory cells fuse to form multicellular complexes in SW-transferred tilapia embryos and larvae, suggesting the multicellular complex to be salt-secreting, SW-type chloride cells. Considering this notion, we conclude that enlarged chloride cells following SW transfer result from enlargement of pre-existing chloride cells and simultaneous formation of cellular complexes together with newly-developed accessory or chloride cells. This is also supported by the *in vivo* observation that the enlarged chloride cells in SW contain two or more DASPEI-negative nuclei. In contrast, chloride cells neither changed their size nor newly formed multicellular complexes in fish kept in FW. These results indicate that FW-type, single chloride cells are transformed into SW-type, chloride cell complexes during SW adaptation, suggesting the excellent plasticity in altering ion-transporting functions of chloride cells.

Chloride cell complexes increased after SW transfer, whereas they decreased in FW. These results also indicate the importance of chloride cell complexes for SW adaptation. In the transmission electron microscopic observation of the yolk-sac membrane of SW-adapted tilapia larvae, Shiraishi *et al.* (1997) have proved that main chloride cells and adjacent accessory cells interdigitate with each other to form multiple junctions. The chloride cell complex is considered to be advantageous to Na^+ secretion, since Na^+ secretion may occur down its electrochemical gradient via a paracellular pathway in the complex (Silva *et al.* 1977; Marshall 1995; McCormick 1995). It should be noted that the considerable number of chloride cell complexes important for SW adaptation are present in FW embryos. The number of the complexes decreases as the fish develop in FW. Our unpublished study shows that the ability of SW adaptation in FW tilapia is most excellent before hatching. FW embryos readily survive direct transfer to SW; however, SW adaptability decrease after hatching, although they are adaptable to SW when transferred gradually. The decrease in SW adaptability during the development could be related to the decrease in chloride cell complexes. The strong euryhalinity of tilapia embryos may be attributed to the occurrence of

SW-type, chloride cell complexes as well as FW-type, single chloride cells in the yolk-sac membrane.

Currently, two types of chloride cells, filament and lamellar chloride cells, are identified in the gills of several teleosts, based on their location and ultrastructural features (Uchida *et al.* 1996; Sasai *et al.* 1998b; Hirai *et al.* 1999). Filament and lamellar chloride cells are considered to be involved in SW and FW adaptation, respectively. Such classification is not applicable to chloride cells in the yolk-sac membrane. However, chloride cells forming multicellular complexes and single chloride cells would correspond to SW-type, filament chloride cells and FW-type, lamellar cells, respectively, in the gills of adult fish.

An increase in the number of chloride cells has been reported in the gills of several teleosts transfer from FW to SW (Pisam and Rambourg 1991). In the present study, the density of chloride cells in the yolk-sac membrane was not different between individuals kept in FW and those transferred to SW. This result is consistent with previous studies with the yolk-sac membrane of the same species (Ayson *et al.* 1994; Shiraishi *et al.* 1997). However, the density of chloride cells in SW individuals may be underestimated because of the formation of chloride cell complex during SW adaptation. Chloride cell complexes increased in SW-transferred fish have been counted as one cell in the present and previous studies. If the precise number of cells in the complex could be determined, the chloride cell number would be greater in SW than in FW.

In both FW and those transferred to SW, a certain proportion of chloride cells were continuously replaced with newly-differentiated cells, providing direct evidence for the occurrence of cellular turnover. The number of both disappearing and newly-appearing cells in FW individuals was significantly larger than in SW individuals, suggesting a higher turnover rate in FW than in SW. In the gill epithelium of chum salmon (*Oncorhynchus keta*) fry, however, a higher rate of chloride

cell turnover has been observed in fish transferred from FW to SW than FW-adapted fish (Uchida and Kaneko 1996). The longevity of chloride cells in SW individuals must be a characteristic of the yolk-sac membrane, or the response of chloride cells to different osmotic conditions could vary between species.

Besides the confirmation of the normality of DASPEI-stained chloride cells, the combination of DIC and immunofluorescence images of chloride cells in the fixed yolk-sac membrane provided us information on the developmental processes of chloride cells and chloride cell complexes. We identified three types of chloride cells: a single chloride cell without an apical pit, a single chloride cell with an apical pit, and a multicellular complex with an apical pit. Since single chloride cells without pits are not exposed to the external environment and smaller in size, they would be immature cells ready to function. Their Na^+, K^+-ATPase immunoreactivity in the cytoplasm indicates that the tubular system has already developed. Those immature chloride cells may originate from undifferentiated cells with a nucleus similar to that of chloride cells. Immature chloride cells would then enlarge their cytoplasm and come into contact with the ambient water by forming apical pits to start functioning as ion-transporting cells.

In individuals transferred to SW, chloride cells with pits were frequently associated with undifferentiated cells. Most of chloride cell complexes consisted of one main chloride cell and one (rarely two) accessory cell. Occasionally, two or more chloride cells of similar size gathered and shared one pit jointly, and this type of the complex was also associated with one or two accessory cells. Accessory cells are thought to be young stages of chloride cells (Sardet *et al.* 1979; Hootman and Philpott 1980; Wendelaar Bonga and van der Meij 1989). The observation on the fixed yolk-sac membrane indicates that accessory cells are also developed from undifferentiated cells attaching the main chloride cells and gradually intrude into the main chloride cells. Most probably, as the main

chloride cells in the complexes degenerate, accessory cells could further develop and replace them. However, the replacement of main chloride cells with accessory cells was not confirmed here, probably because the experimental period of 96 h was too short to observe the cellular turnover in the complex.

Although the role of Na^+ and Cl^- excretion in chloride cells of SW fish is well established (Foskett and Scheffey 1982), their role in the uptake of Na^+ and Cl^- in FW fish is still controversial. We have recently demonstrated that the immunoreactivity of vacuolar-type H^+-ATPase, which generates a favorable electrochemical gradient for passive electrodiffusion of Na^+ through a Na^+ channel (Lin and Randall 1995), is not detected in chloride cells but in pavement cells in the yolk-sac membrane of FW tilapia larvae (Hiroi *et al.* 1998b). Therefore, pavement cells, but not chloride cells, seem to be the site for Na^+ uptake in tilapia yolk-sac membrane. As suggested in the gills of adult fish (Flik *et al.* 1995), FW-type, single chloride cells in the yolk-sac membrane of tilapia embryos and larvae are most likely the site of Ca^{2+} uptake.

Chapter 4: Immunolocalization of Vacuolar-Type H^+-ATPase in the Yolk-Sac Membrane of Tilapia (*Oreochromis mossambicus*) Larvae

ABSTRACT—To investigate the involvement of the yolk-sac membrane in ion absorption, developmental changes in whole-body cation contents, cellular localization of vacuolar-type H^+-ATPase (V-ATPase), and size and density of pavement and chloride cells in the yolk-sac membrane were examined in tilapia (*Oreochromis mossambicus*) larvae in fresh water (FW) and those transferred to seawater (SW) at 2 days before hatching (day –2). The whole-body content of Na^+ in embryos and larvae adapted to both FW and SW increased constantly from day –2 to day 10, although they were not fed through the experiment. The yolk-sac membrane of FW larvae at days 0 and 2 showed V-ATPase immunoreactivity in pavement cells, but not in chloride cells. No positive immunoreactivity was detected in SW larvae. Whole-mount immunocytochemistry showed that some pavement cells were intensively immunoreactive, whereas others were less or not immunoreactive. Electron-microscopic immunocytochemistry revealed that V-ATPase immunoreactivity was present in the apical regions of pavement cells in FW larvae, especially in their ridges. The pavement cells in FW larvae were significantly smaller in size but higher in density than those in SW. These results suggest that pavement cells are the site of active Na^+ uptake in exchange for H^+ secretion through V-ATPase in FW-adapted tilapia during early life stages.

INTRODUCTION

Freshwater (FW) teleosts take up ions from dilute environments across the gill epithelium, compensating for the constant loss of ions by diffusion (Lin and Randall 1995; Goss *et al.* 1995;

Flik *et al.* 1995). It is suggested that the branchial Na^+ uptake occurs through a Na^+ channel electrically coupled with a vacuolar-type H^+-ATPase (V-ATPase), which generates a favorable electrochemical gradient for passive electrodiffusion of Na^+ through a Na^+ channel (Lin and Randall 1995). Previous studies using rainbow trout (*Oncorhynchus mykiss*) gills have suggested that V-ATPase immunoreactivity is distributed in both chloride and pavement cells (Lin *et al.* 1994) or in pavement cells (Sullivan *et al.* 1995). However, cellular localization of V-ATPase and its involvement in Na^+ uptake are still controversial in teleosts.

Embryos and larvae without functional gills can also maintain internal ion concentrations (Alderdice 1988). The gills occupy a large proportion of the body surface in adult form of fish, whereas the epithelia covering the body and yolk are the major part of the body surface in early life stages. The outermost layer of these epithelia consists of pavement cells and, to a less extent, chloride cells. Thus, these cell types in the body skin and yolk-sac membrane could be the site of ion regulation in embryos and yolk-sac larvae, which lack functional gills. In fact, chloride cells in the body skin and yolk-sac membrane have been suggested to be the site of salt secretion in seawater (SW)-adapted embryos and larvae of several teleosts (Shelbourne 1957; Lasker and Threadgold, 1968; Hwang and Hirano 1985; Alderdice 1988; Hwang 1989, 1990; Ayson *et al.* 1994; Kaneko *et al.* 1995; Shiraishi *et al.* 1997). However, little information is available on ion uptake mechanisms in FW-adapted embryos and larvae.

In the present study, we examined changes in whole-body cation contents during the early development of euryhaline tilapia (*Oreochromis mossambicus*) in FW and those adapted to SW. To explore the possible involvement of the yolk-sac membrane in ion absorption, cellular localization of V-ATPase was also examined in FW and SW fish, concomitant with morphometrical analyses of pavement and chloride cells. Our findings suggest that Na^+ uptake occurs in pavement

cells of the yolk-sac membrane in FW-adapted tilapia during early life stages, when functional gills are lacking.

MATERIALS AND METHODS

Fish

Mature tilapia (*Oreochromis mossambicus*) were maintained in tanks with recirculating FW (Na^+, 0.74 mM; Ca^{2+}, 0.54 mM; Mg^{2+}, 0.26 mM) at 25°C. Fertilized eggs were obtained from the mouth of brooding females at 3 days after fertilization (2 days before hatching, day –2). They were separated into two groups: half of the embryos were maintained in FW, and the other half were transferred directly to SW (Na^+, 490 mM; Ca^{2+}, 16 mM; Mg^{2+}, 66 mM) at day –2. They were further incubated in a 5-l plastic tank containing FW or SW at 25°C. Water was gently agitated with aeration and renewed every other day. Tilapia embryos usually hatched after 5 days' incubation at 25°C, and yolk absorption was completed by 10 days after hatching. Larvae were not fed during the experiment.

Whole-body cation contents

FW embryos at day –2 and FW and SW larvae at days 0, 2, 5 and 10 were rinsed with distilled water, weighed and preserved at –20°C. The chorion of embryos was torn off with sharp-pointed forceps. Whole-body cation contents were measured following the method of Uchida *et al.* (1993) with some modifications. Each sample was dissolved with 500 µl of a mixture of nitric acid and perchloric acid (11:2). The concentrations of Na^+, Ca^{2+} and Mg^{2+} were measured with an atomic absorption spectrophotometer (Hitachi 180-50, Japan).

Semi-thin section immunocytochemistry

FW and SW larvae at days 0 and 2 were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 20 h at 4˚C, dehydrated through graded ethanols, transferred to propylene oxide and embedded in Spurr's resin. Cross sections were cut at 1 μm thickness with glass knives, and mounted on slides coated with Biobond adhesive (British BioCell, UK). The sections were treated with saturated potassium hydroxide in ethanol for 10 min to remove the resin, and then subjected to immunocytochemical staining for V-ATPase based on the silver enhancement method (Springall *et al*. 1984). The sections were incubated sequentially with: 1) 2% normal goat serum in 0.01 M phosphate-buffered saline (PBS, pH 7.2) for 30 min, 2) rabbit anti-V-ATPase diluted 1:2000 for 20 h at 4˚C, and 3) goat anti-rabbit IgG conjugated with 5 nm-immunogold (British BioCell, UK) diluted 1:100 for 2 h. The immunoreaction was visualized by treating the sections with the silver enhancing reagent (British BioCell, UK) for 10 min at 22˚C. The antiserum was diluted with PBS containing 2% normal goat serum, 0.1% bovine serum albumin and 0.01% sodium azide (BPBS). The sections were then washed with distilled water, coverslipped with distilled water, and examined with a microscope equipped with a Nomarski's differential interference contrast (DIC) device. Mounting medium was not used because it reduced the contrast of DIC images.

Whole-mount immunocytochemistry

After FW and SW larvae at day 2 were fixed in 4% paraformaldehyde in PB for 30 min, the yolk sac was incised and the yolk was carefully scraped out with sharp-pointed forceps. Larvae were further fixed for 6 h and preserved in 70% ethanol. Whole-mount immunocytochemistry

based on the avidin-biotin-peroxidase complex (ABC) method (Hsu *et al*. 1981) with commercial reagents (Vectastain ABC kit, Vector Laboratories, USA) was carried out following the method of Ohtani *et al*. (1989). After treatment with 0.1% sodium cyanoborohydride in PBS for 1 h, the samples were incubated sequentially with: 1) rabbit anti-V-ATPase diluted 1:500 for 20 h at 4˚C, 2) biotinylated goat anti-rabbit IgG for 20 h at 4˚C, 3) ABC reagent for 20 h at 4˚C, and 4) 0.03% 4-Cl-1-naphthol in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.003% hydrogen peroxide for 20 min. The antiserum was diluted with PBS containing 0.05% Triton X-100, 10% normal goat serum, 0.1% bovine serum albumin and 0.01% sodium azide. The yolk-sac membrane was then removed, mounted on a slide with glycerin, and examined with a microscope equipped with a Nomarski's DIC device.

Electron-microscopic immunocytochemistry

FW and SW larvae at day 2 were fixed in 2% paraformaldehyde-0.2% glutaraldehyde in PB for 3 h at 4˚C and embedded in Spurr's resin. Ultra-thin sections were cut with a diamond knife and mounted on nickel grids. The sections were incubated sequentially with: 1) BPBS for 5 min, 2) rabbit anti-V-ATPase diluted 1:1000 with BPBS for 2 h, and 3) goat anti-rabbit IgG conjugated with 10 nm-immunogold (British BioCell, UK) diluted 1:50 with PBS for 1hr. All procedures were performed at room temperature. The sections were then stained with uranyl acetate (10 min) and lead citrate (1 min), and examined with a transmission electron microscope (Hitachi H-7100, Japan).

Antiserum

The polyclonal antibody used here was raised in a rabbit against a V-ATPase 72 kDa subunit A isolated from chromaffin granule membranes in bovine adrenal medulla (Moriyama and

Yamamoto 1995). The antiserum was kindly provided by Professor Yoshinori Moriyama, Institute of Scientific and Industrial Research, Osaka University. To confirm the specificity of the immunoreactivity, the primary antiserum was replaced with normal rabbit serum at the same dilution, which resulted in extinction of the immunoreaction.

Morphometrical analyses of pavement and chloride cell

FW and SW larvae at day 2 were fixed in the same procedure as the whole-mount immunocytochemistry, and then postfixed in 1% osmium tetroxide in PB for 1 h. After the larvae were washed with distilled water, the yolk-sac membrane was removed, mounted on a slide with distilled water, and examined with a microscope equipped with a Nomarski's DIC device. The images of 10 fields, corresponding to 0.274 mm^2, from each individual were digitized with a CCD video camera (Victor, Japan) and an image processor (ARGUS 20, Hamamatsu photonics, Japan), and the area and density of pavement and chloride cells were measured on an Apple Macintosh computer using the public domain NIH Image program (available on the Internet at http://rsb.info.nih.gov/nih-image/).

Statistics

Significant differences in whole-body cation contents were examined by two-way ANOVA with repetition (time x environment) followed by the Scheffe's test. Before analysis, data displaying heterogeneity of variance were log-transformed. Significant differences in size and density of pavement and chloride cells between FW and SW groups were tested by the Kolmogorov-Smirnov test and the Mann-Whitney test, respectively.

RESULTS

Whole-body cation contents

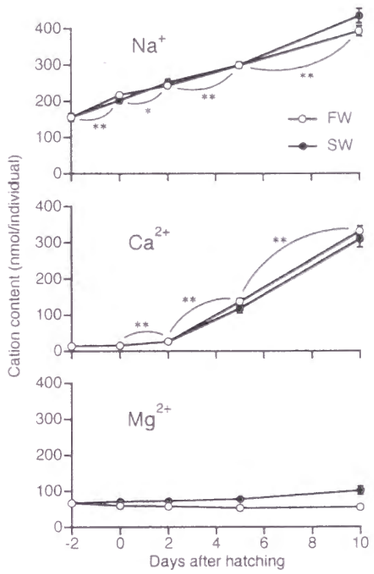


Fig. 1. Developmental changes in whole-body cation contents. Vertical bars represent standard errors of the means of 10 individuals. Asterisks indicate significant differences by the Scheffe's test. $*P < 0.01$, $**P < 0.001$.

Developmental changes in whole-body cation contents are shown in Figure 1. The Na^+ content of embryos and larvae adapted to both FW and SW increased constantly from day –2 to day 10. The whole-body Ca^{2+} content of both groups kept a constant level before hatching, doubled at day 2, and increased rapidly thereafter. In contrast, the Mg^{2+} content of both groups showed no significant change during the experiment. There were no significant differences in the three cation contents between FW and SW.

Semi-thin section and whole-mount immunocytochemistry

In the semi-thin section immunocytochemistry, V-ATPase immunoreactivity was detected in the yolk-sac membrane of FW larvae at days 0 and 2, whereas the positive immunoreactivity was

not detectable in SW larvae at days 0 and 2 (Fig. 2). The DIC images allowed to distinguish between pavement and chloride cells. Cone-like shaped chloride cells were in contact with the basal membrane on the serosal side, and with external environments on their apical membrane forming a pit. In FW larvae, V-ATPase immunoreactivity was restricted to pavement cells, and was not detected in chloride cells (Fig. 2A). However, V-ATPase immunoreactivity was not observed in all pavement cells in FW larvae, which was further confirmed by the whole-mount immunocytochemistry. Figure 3 shows whole-mount preparations of the yolk-sac membrane of FW and SW larvae at day 2, stained with anti-V-ATPase. Some pavement cells in FW larvae showed strong immunoreactivity, whereas others showed little or no immunoreactivity (Fig. 3A). Chloride cells, readily distinguishable from pavement cells by changing the focus, showed no immunoreactivity. In addition, no positive immunoreactivity was detected in pavement and chloride cells of SW larvae (Fig. 3B).

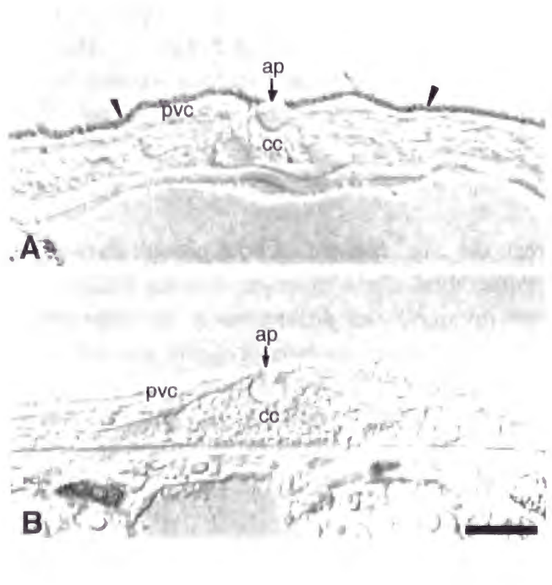


Fig. 2. DIC micrographs of the yolk-sac membrane stained with anti-V-ATPase in larvae (day 2) adapted to FW (**A**) and SW (**B**). V-ATPase immunoreactivity (*arrowheads*) is detectable in pavement cells (*pvc*), but not in chloride cells (*cc*), in FW larvae. Both pavement and chloride cells are immunonegative in SW larvae. *ap*, apical pit of chloride cells. Bar: 10 µm.

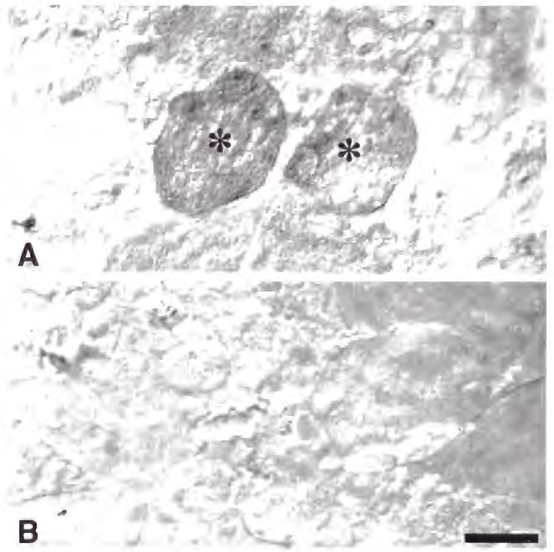


Fig. 3. Whole-mount preparations of the yolk-sac membrane stained with anti-V-ATPase in larvae (day 2) adapted to FW (**A**) and SW (**B**). Some pavement cells are immunoreactive (*asterisks*) in FW larvae, while no immunoreactivity is detectable in SW larvae. Bar: 20 µm.

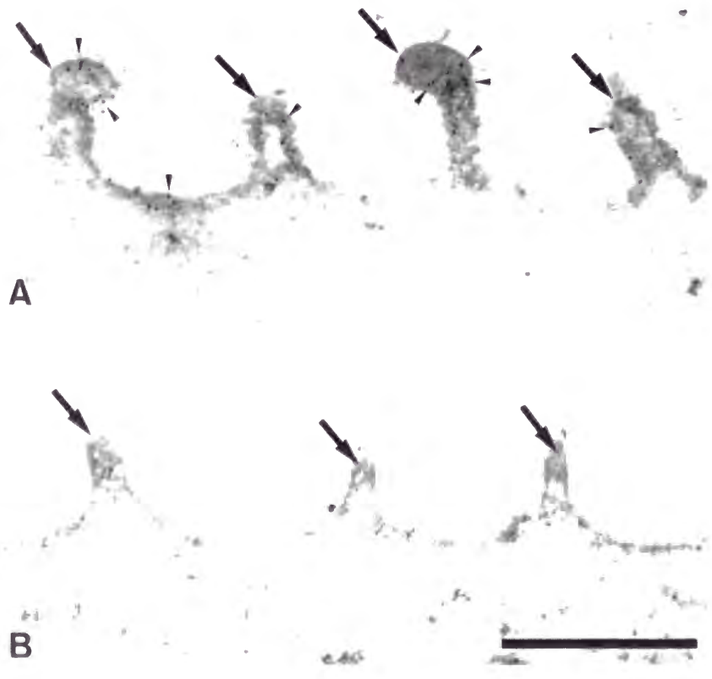


Fig. 4. Transmission-electron micrographs of pavement cells in the yolk-sac membrane stained with anti-V-ATPase in larvae (day 2) adapted to FW (**A**) and SW (**B**). Immunogold labeling (*arrowheads*) is mainly located in the apical regions of pavement cells, especially in the ridge structures (*arrows*), in FW larvae. Pavement cells in SW larvae are immunonegative. Bar: 1 µm.

Electron-microscopic immunocytochemistry

Figure 4 shows transmission-electron micrographs of the yolk-sac membrane of FW and SW larvae at day 2. In FW larvae, V-ATPase immunogold labeling was present in the apical regions of

the pavement cells, especially in the ridge structures (Fig. 4A). In accordance with the light-microscopic observations, no immunogold labeling was detected in SW larvae (Fig. 4B).

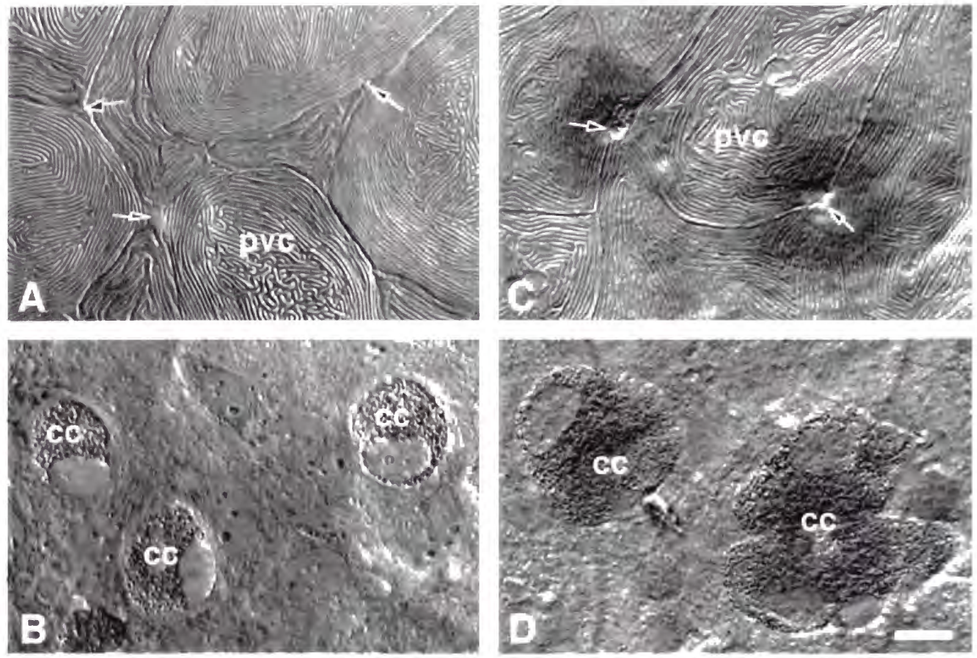


Fig. 5. DIC micrographs of whole-mount preparations of the yolk-sac membrane in larvae (day 2) adapted to FW (**A**, **B**) and SW (**C**, **D**). The ridge structures of pavement cells (*pvc*) and apical pits (*arrows*) of chloride cells (*cc*) are observable on the surface (**A**, **C**), while the underlying chloride cells are in focus on a deeper plane (**B**, **D**) of the same specimens. Bar: 10 μm

Size and density of pavement and chloride cells

In the whole-mount preparations of the yolk-sac membrane, the ridge structures of pavement cells and apical pits of chloride cells were clearly observed under a light microscope with a DIC device (Figs. 5A, C). In the same specimen, chloride cells were also observable beneath the pits by changing the focus (Figs. 5B, D). The pavement cells were significantly smaller ($P < 0.001$) in FW larvae than those in SW (Fig. 6). The density of pavement cells in FW larvae was 1925 ± 253 cells/mm^2 (mean ± SE, n = 4), which was significantly higher ($P < 0.05$) than those in SW (823

± 125 cells/mm^2). The chloride cells were significantly larger ($P < 0.001$) in SW larvae than in FW larvae (Fig. 7), whereas there was no significant difference in the density of chloride cells between the two groups (FW, 462 ± 42 cells/mm^2; SW, 458 ± 50 cells/mm^2).

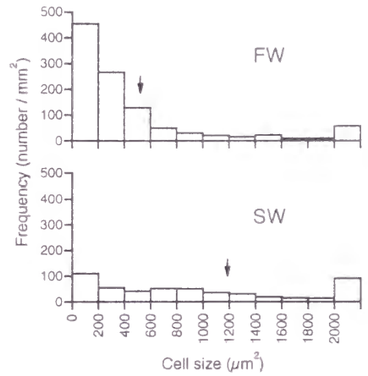


Fig. 6. Size-frequency distributions of pavement cells in the yolk-sac membrane of larvae (day 2) adapted to FW and SW. Data obtained from 4 individuals were combined in each group. Arrows indicate the means.

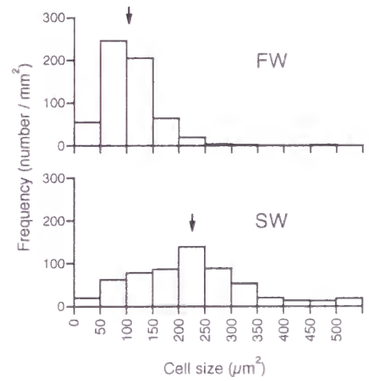


Fig. 7. Size-frequency distributions of chloride cells in the yolk-sac membrane of larvae (day 2) adapted to FW and SW. Data obtained from 4 individuals were combined in each group. Arrows indicate the means.

DISCUSSION

In the present study, a constant increase in Na^+ content was demonstrated during the early development of tilapia adapted to FW and SW. A rapid increase in Ca^{2+} content occurred at day 2, whereas Mg^{2+} content was essentially constant throughout the experiment. Hwang *et al.* (1994) observed similar increases in Na^+ and Ca^{2+} contents in FW-adapted tilapia embryos and larvae.

These findings indicate that tilapia uptake Na^+ and Ca^{2+} from external environments during the early developmental stages. According to Miyazaki *et al.* (1998), FW-adapted tilapia larvae start drinking at day 2. The gills are not yet developed or not fully functional during embryonic and early larval stages (Ayson *et al.* 1994; Li *et al.* 1995). Taken together, Na^+ and Ca^{2+} uptakes are expected to occur mainly across the body surface during early life stages, when functional gills are lacking.

In the early life stages of tilapia, the yolk-sac membrane covering the yolk occupies a large proportion of the body surface, which contrasts with the large surface area of the gills in adult fish. The surface structure of the yolk-sac membrane is morphologically similar to that of the gills: the outermost layers of both tissues are covered with a single layer of respiratory pavement cells, and chloride cells are scattered among the pavement cells with their pits exposing to external environments. Thus, it may be reasonable to presume that the yolk-sac membrane functions as a respiratory and ion-regulating site in embryos and yolk-sac larvae in place of the gills in adult fish.

In FW fish gills, an electroneutral Na^+/H^+ exchanger in the apical membrane had been advocated as the major pathway for Na^+ uptake and H^+ secretion. However, the Na^+/H^+ exchanger model was questioned by experimental and theoretical evidence, and an alternative model incorporating an electrogenic proton pump and a conductive Na^+ channel was proposed on the analogy of other epithelia such as amphibian skin (Avella and Bornancin 1989). In this scheme, V-ATPase, an electrogenic proton pump, generates a favorable electrochemical gradient across the apical membrane and permits passive electrodiffusion of Na^+ through a Na^+ channel. The presence of V-ATPase and its mRNA has been reported in rainbow trout gills (Lin *et al.* 1994; Sullivan *et al.* 1995, 1996). Sullivan *et al.* (1995) have concluded that V-ATPase is present in pavement cells in rainbow trout gills. Morphological observations and X-ray microanalysis also suggest that pavement cells are the site of Na^+ uptake and H^+ secretion (Goss *et al.* 1992, 1994; Laurent *et al.*

1994; Morgan *et al.* 1994). However, the occurrence of V-ATPase in chloride cells in addition to pavement cells is still controversial, partly because of the difficulty in identifying chloride cells. In the yolk-sac membrane of tilapia embryos and larvae, we were able to distinguish between pavement and chloride cells under a light microscope without difficulty, and demonstrated V-ATPase immunoreactivity only in pavement cells in FW larvae. Furthermore, the electron-microscopic immunocytochemistry revealed that V-ATPase was localized in the apical regions of pavement cells. These observations suggest that pavement cells in the yolk-sac membrane are the site of Na^+ uptake in exchange for H^+ secretion in FW-adapted tilapia larvae. This is also supported by the absence of V-ATPase immunoreactivity in SW-adapted fish.

The V-ATPase and Na^+ channel model in FW fish is based on a model established in the amphibian skin. V-ATPase is apically located in mitochondria-rich (MR) cells of the amphibian skin, which are responsible for H^+ secretion. However, MR cells are not the principal site of Na^+ transport, but granular cells are responsible for Na^+ transport (Brown and Breton 1996; Nagel and Dörge 1996). Since the localization of Na^+ channel was not demonstrated in the yolk-sac membrane of tilapia larvae, we are not able to conclude that V-ATPase-immunopositive pavement cells are the site of Na^+ uptake. The whole-mount immunocytochemistry revealed that pavement cells were either immunopositive or -negative to V-ATPase. It is possible that Na^+ channel might be present in a subpopulation of pavement cells which are immunonegative to V-ATPase.

The surface structures of pavement cells and apical openings of chloride cells have been observed in the yolk-sac membrane and skin of tilapia embryos and larvae by scanning-electron microscopy (Ayson *et al.* 1994; Hwang *et al.* 1994; Shiraishi *et al.* 1997). Although the scanning microscopy provides information on fine surface structures of epithelia at high magnifications, chloride cells existing beneath the apical pits are not observable. In the present study, we

successfully observed not only ridge structures of pavement cells and apical pits of chloride cells on the surface, but also the underlying chloride cells on a deeper plane in the same yolk-sac membrane preparations with a light microscope.

Pavement cells in the yolk-sac membrane were smaller but more numerous in FW larvae than in SW larvae. There is no difference between FW and SW in total area of the yolk-sac membrane, which is mostly covered with pavement cells. Thus, unlike chloride cells that are sparsely distributed in the yolk-sac membrane, the mean size and density of pavement cells are in inverse proportion to each other. The larger number and smaller size of pavement cells in FW than in SW might be interpreted as enhanced cellular activity in FW. It might also be possible that more frequent recruitment of newly-differentiated pavement cells in FW results in the large number and small size of these cells.

Chloride cells in the yolk-sac membrane were larger in SW larvae than in FW larvae, whereas there was no difference in density between FW and SW. These findings accord with observations by Ayson *et al.* (1994), who detected chloride cells in the yolk-sac membrane by means of DASPEI staining in FW- and SW-adapted tilapia embryos and larvae. According to Shiraishi *et al.* (1997), well-developed chloride cells in SW tilapia embryos and larvae form multicellular complexes, consisting of chloride cells and accessory cells. Chloride cells, or chloride cell complexes, in the yolk-sac membrane of SW-adapted fish are considered to be the site for salt secretion. On the other hand, the functional significance of chloride cells in the yolk-sac membrane in FW-adapted fish still remains unknown. In adult fish, gill chloride cells appear to be responsible for Ca^{2+} uptake in FW (Flik *et al.* 1995). Compared with chloride cells in SW larvae, those in FW were small but equally numerous, suggesting that chloride cells in FW play an ion-transporting role(s) different from that in SW. The observed increase in Ca^{2+} content during the early development of FW tilapia might be due

to chloride cells in the yolk-sac membrane, although no direct evidence for this issue is available at present.

In summary, V-ATPase immunoreactivity was demonstrated in pavement cells of the yolk-sac membrane of tilapia larvae adapted to FW. A constant increase in Na^+ content during the early development, the immunolocalization of V-ATPase in pavement cells, and higher density of pavement cells in FW suggest that pavement cells are the site responsible for active Na^+ uptake in exchange for H^+ secretion in FW-adapted embryos and larvae.

Concluding Remarks

A large number of marine teleosts undergo physiological changes as well as morphological and ecological changes during metamorphosis from larvae to juveniles. In Japanese flounder, an increase in low-salinity tolerance was observed during metamorphosis, coinciding with ecologically-observed migration to near-shore and estuarine areas. The prolactin cell volume of the larvae reared in SW increased gradually during metamorphosis and markedly after low-salinity transfer. These results suggest that prolactin is involved in the development of low-salinity tolerance during metamorphosis of the flounder. Whole-body concentrations of thyroid hormones decreased slightly but significantly after low-salinity transfer, also suggesting possible involvement of thyroid hormones in low-salinity adaptation of the flounder. No significant difference in whole-body concentration of cortisol was observed after low-salinity transfer. The extraction method established in the present study would be useful for future endocrine studies with fish embryos and larvae.

A spatial shift of chloride cell distribution from the body skin to the gills was clearly observed during metamorphosis of the flounder by whole-mount immunocytochemistry with anti-Na^+, K^+-ATPase. Since many other marine teleosts also exhibit metamorphosis to varying degree, the shift from cutaneous to branchial chloride cells could be expected to occur during metamorphosis in those teleosts. The occurrence of multicellular complexes of chloride cells in the body of premetamorphic flounder larvae suggests that those cells function as ion-secreting sites in seawater. Although flounder larvae and juveniles are not adaptable to FW, they can survive in hypotonic environments to a certain extent. During low-salinity adaptation, chloride cells in the body skin and gills may function as the site of ion uptake, which could be under the endocrine control of prolactin.

The appearance of branchial chloride cells before the differentiation of gill lamellae suggests that the primary function of the gills during the early development is ion regulation rather than gas exchanges.

Since Keys and Willmer (1932) first identified and named "chloride secreting cell", many studies have been carried out on chloride cells in the gills. However, the complex, three-dimensional structures of the gills, together with their poor viability under *in vitro* conditions, have put difficulties in examining branchial ion transport mechanisms. The simple structure of the yolk-sac membrane of tilapia and the combination of DASPEI staining and confocal laser scanning microscopy enabled me to observe sequential changes in individual chloride cells during seawater adaptation. The *in vivo* observation system established here indicated that a large proportion of chloride cells survived after seawater transfer, and that those cells increased markedly in size. This system would be effective in examining direct effects of hormones and environmental ion concentrations on chloride cell morphology in future studies. The combination of whole-mount immunocytochemistry with anti-Na^+, K^+-ATPase and differential interference contrast (DIC) optics allowed to classify chloride cells into three developmental stages: a single chloride cell without an apical pit, a single chloride cell with an apical pit, and a multicellular complex of chloride cells with a common apical pit. Wendelaar Bonga and van der Meij (1989) reported five (accessory, immature, mature, apoptotic or necrotic) developmental stages of chloride cells in the gills of tilapia, based on their transmission electron microscopical observations. While transmission electron microscopy provides fine structures of chloride cells, immunofluorescence and DIC observations on whole-mount preparations help us to understand the whole image of chloride cells. Immunofluorescence and DIC observations also revealed that single chloride cells were enlarged and frequently dented by newly-differentiated accessory cells to form multicellular complexes during SW adaptation. These

results indicate that FW-type, single chloride cells are transformed into SW-type, chloride cell complexes during SW adaptation, suggesting the excellent plasticity in altering ion-transporting functions of chloride cells in the yolk-sac membrane of tilapia embryos and larvae.

During the early development of tilapia adapted to FW and SW, a constant increase in Na^+ content was demonstrated. A rapid increase in Ca^{2+} content occurred at day 2, whereas Mg^{2+} content was essentially constant throughout the experiment. These findings indicate that tilapia uptake Na^+ and Ca^{2+} from external environments during the early developmental stages. The yolk-sac membrane seems to function as a respiratory and ion-regulating site in embryos and yolk-sac larvae in place of the gills in adult fish. In FW fish gills, an electroneutral Na^+/H^+ exchanger in the apical membrane had been advocated as the major pathway for Na^+ uptake and H^+ secretion. However, the Na^+/H^+ exchanger model was questioned by experimental and theoretical evidence, and an alternative model incorporating an electrogenic proton pump and a conductive Na^+ channel has been proposed on the analogy of other epithelia such as amphibian skin (Avella and Bornancin 1989). In this scheme, V-ATPase, an electrogenic proton pump, generates a favorable electrochemical gradient across the apical membrane and permits passive electrodiffusion of Na^+ through a Na^+ channel. In the yolk-sac membrane of tilapia embryos and larvae, I succeeded in distinguishing between pavement and chloride cells under a light microscope without difficulty, and demonstrated V-ATPase immunoreactivity only in pavement cells in FW larvae. Furthermore, the electron-microscopic immunocytochemistry revealed that V-ATPase was localized in the apical regions of pavement cells. These observations suggest that pavement cells in the yolk-sac membrane are the site for Na^+ uptake in exchange for H^+ secretion in FW-adapted tilapia larvae. This is also supported by the absence of V-ATPase immunoreactivity in SW-adapted fish. The larger number and smaller size of pavement cells in FW than in SW might be interpreted as enhanced

cellular activity in Na^+ uptake in FW. It might also be possible that more frequent recruitment of newly-differentiated pavement cells in FW results in the large number and small size of these cells. It should be noted that a higher rate of chloride cell turnover was also observed in FW than in SW in Chapter 3. In several teleosts, a higher turnover rate of gill epithelial cells has been observed in SW than in FW (Conte and Lin 1967; Chretien and Pisam 1986). The longevity of both pavement and chloride cells in SW individuals must be a characteristic of the yolk-sac membrane, or the response of these cells to different osmotic conditions could vary between species.

In summary, prolactin and thyroid hormones seem to be involved in low-salinity adaptation of Japanese flounder during metamorphosis and inshore migration. Cutaneous chloride cells decreased in size and density and disappeared during metamorphosis of Japanese flounder, whereas branchial chloride cells increased in number during metamorphosis. These results indicate that the site for ion secretion in seawater shift from cutaneous to branchial chloride cells during metamorphosis. In the yolk-sac membrane of tilapia embryos and larvae, FW-type, single chloride cells are transformed into SW-type, chloride cell complexes during SW adaptation, suggesting the excellent plasticity in altering ion-transporting functions of chloride cells. The pavement cells in the yolk-sac membrane of FW tilapia larvae showed V-ATPase immunoreactivity, but not in chloride cells. Pavement cells would be the site of active Na^+ uptake in exchange for H^+ secretion through V-ATPase in FW-adapted tilapia during early life stages.

Finally, I would like to emphasize that chloride and pavement cells in the yolk-sac membrane and body skin are not only important in osmotic and ionic regulation in the early life stages of teleost species, but also excellent models for future studies in search of chloride and pavement cell functions in general, because of their simple structures.

要旨

硬骨魚類の浸透圧調節機構については，サケ科魚類などの成魚を材料として多くの研究がなされてきた．淡水適応にはプロラクチン，海水適応にはコルチゾルや成長ホルモン等のホルモンが関与していること，鰓，腎臓および消化管がこれらのホルモンの標的器官であり重要な浸透圧調節器官であること，その中でも鰓に存在する塩類細胞が主要なイオン調節部位であること，などがこれまでの研究で明らかにされている．しかし，これらの浸透圧調節器官が未発達な発育初期における浸透圧調節機構については知見が少ない．特に硬骨魚類の中で多数を占める海産魚の仔魚期における浸透圧調節機構に関する研究はほとんどなされていないのが現状である．こうした状況を踏まえて，本研究では水産学上重要な魚種であるヒラメおよび優れた広塩性を示し浸透圧調節機構を調べる上で好適な実験材料となるティラピアを用い，発育初期における浸透圧調節機構の解明を目指した．

まず第1章では，海産魚の代表としてヒラメをとりあげ，仔稚魚期における低塩分適応能力の変化と内分泌系の関与について検討した．さらに第2章では，ヒラメ仔魚の発育に伴う塩類細胞の存在部位の遷移の様子を明らかにした．

成魚の鰓の塩類細胞は，淡水中では環境水からのイオンの取り込みに関与しており，海水中では体内に過剰となったイオンの排出の場であると考えられている．しかし，淡水から海水への移行の際に鰓の塩類細胞がどのような挙動を示すのかについては，複雑で繊細な構造を持つ鰓の実験材料としてのデメリットも災いして未だ明らかにされていない．そこで第3章では，広塩性魚類ティラピアの胚を淡水から海水に移行した際に個々の塩類細胞の変化をin vivoで連続的に観察することによって，海水適応時における塩類細胞の動態を明らかにした．また，海水適応時におけるイオンの排出機構に比べて，淡水適応時におけるイオンの取り込み機構については知見が乏しい．そこで第4章では，ティラピア仔魚が淡水適応時に環境水からNa^+とCa^{2+}を積極的に取り込んでいることを明らかにすると共に，Na^+の取り込み機構について考察した．

第1章　ヒラメの仔稚魚期における低塩分耐性の発現と浸透圧調節ホルモンの関与

ヒラメ（*Paralichthys olivaceus*）は仔魚から稚魚への移行期に相当する変態期に沖合域から河口周辺域への回遊（接岸回遊）を行うが，このような生息場所の変化に備えて変態期に低塩分耐性を獲得することが予測される．そこで，通常海水中で卵から飼育したヒラメを発育ステージが進むごとに希釈海水に移行して生残率を調べたところ，孵化直後の卵黄仔魚は比較的低塩分に強いが，その後低塩分耐性は低下し，変態期になると再び上昇することが明らかになった．また，通常海水中で飼育した仔稚魚の各発育ステージにおいて，成魚において淡水適応ホルモンとされているプロラクチンの脳下垂体における産生状態を免疫染色を用いて調べたところ，変態の進行に伴ってプロラクチン産生能力が上昇することが明らかとなった．したがって，変態期における低塩分耐性の上昇は，プロラクチン産生能力の上昇と深く関連していると考えられた．次に，前変態期，変態最盛期および稚魚期のヒラメを通常海水から希釈海水に移行し，プロラクチンの産生状態と甲状腺ホルモンおよびコルチゾル体内濃度の48時間後の変化を免疫染色およびラジオイムノアッセイ法を用いて測定したところ，どの発育段階においても希釈海水移行後にプロラクチンの産生が顕著に上昇したのに対して，甲状腺ホルモンおよびコルチゾル体内濃度は有意な変化を示さなかった．したがって，プロラクチンはヒラメの仔稚魚期を通して低塩分適応ホルモンとして働いていることが強く示唆された．

第2章　ヒラメの変態期における塩類細胞の体表から鰓への分布の遷移

海産魚の成魚では体液中に過剰となるNa^+およびCl^-を鰓に存在する塩類細胞から排出しているとされているが，鰓が未発達な胚期・仔魚期では卵黄嚢および体表に塩類細胞が分布していることがいくつかの魚種において報告されている．これらの卵黄嚢・体表の塩類細胞は発育の進行に伴って消失し鰓の塩類細胞にとって代わられると容易に想像出来るが，その様子を観察した例はこれまでにない．そこで，塩類細胞におけるイオン輸送に重要な役割を果たしているNa^+,K^+-ATPaseに対する抗体を用いたwhole mount免疫染色によって，ヒラメ仔魚の卵黄嚢，体表および鰓に存在する塩類細胞を特異的に検出した．前変態期（ふ化後0 - 18日）の仔魚の卵黄嚢および体表には大型の塩類細胞が多数分布していたが，変

態始動期（ふ化後21，24日）になると体表の塩類細胞の大きさと密度はともに急激に減少し，変態最盛期（ふ化後28，33日）には体表に塩類細胞は検出されなかった．一方，鰓の塩類細胞はふ化後8日に出現し，発育の進行に伴って発達した．これらの結果は，ヒラメにおいて体表から鰓へのイオン調節部位の転換が変態期に起こることを示している．また，鰓ではガス交換に重要な二次鰓弁が分化する以前に塩類細胞が出現したことから，発育初期における鰓の主な機能はガス交換よりもむしろイオンの排出であることが示唆された．

第3章　ティラピア胚仔魚の卵黄嚢上皮に存在する塩類細胞の海水適応時における経時変化

淡水産の広塩性魚類であるティラピア（*Oreochromis mossambicus*）は発生の初期段階から優れた広塩性を示し，胚を淡水から海水へ直接移行しても正常に発生が進行する．ティラピア胚・仔魚の卵黄嚢上皮には多数の塩類細胞が存在し，淡水中では小型の塩類細胞が単独に分布しているのに対して，海水中では塩類細胞は大型化し，複数の細胞が複合体を形成していることが報告されている．成魚の鰓の塩類細胞と同じように前者はイオンの取り込み，後者はイオンの排出に関与していると考えられている．しかし，ティラピア胚を淡水から海水に移行した際に塩類細胞に生じる変化を観察した例はこれまでにない．海水に移行すると淡水中で存在した塩類細胞は消失して新たな塩類細胞が分化してくるのか，それとも淡水中で存在した塩類細胞は海水移行後も残り，形態・機能を変化させて海水タイプの塩類細胞として働くのであろうか．この問いに答えるために，ティラピア胚の卵黄嚢上皮の塩類細胞を蛍光色素で生体染色し，淡水から海水に移行した後の個々の塩類細胞の変化を*in vivo*で経時的に観察した．このような実験は成魚の鰓では不可能であるが（複雑な構造のため器官培養下では観察が困難であり，細胞培養系では塩類細胞が消失してしまう），本研究では単純な平面構造を呈するティラピア胚の卵黄嚢上皮を材料として用い，生体染色と共焦点レーザースキャン顕微鏡を組み合わせることによって個々の塩類細胞の変化を連続的に追うことに成功した．その結果，海水移行4日後の時点で観察開始時の80%に相当する数の塩類細胞が残っており，しかも個々の塩類細胞は4日後に約2倍の大きさになった．一方，対照群として淡水に4日間保持した個体では海水群とほぼ同数の塩類

細胞が残っていたが，個々の塩類細胞の大きさには変化はみられなかった．また，固定したサンプルにNa^+,K^+-ATPase抗体を用いた免疫染色を施し，塩類細胞の構造を高倍率で観察したところ，海水に移行すると塩類細胞に隣接する未分化細胞がアクセサリー細胞に分化し，塩類細胞に陥入して細胞複合体を形成することが明らかとなった．これらの結果より，ティラピアの卵黄嚢上皮において淡水中で存在する塩類細胞は海水移行後も残り，形態・機能を変化させて海水タイプの塩類細胞に変化することが示された．また，いくつかの魚種の稚魚・成魚において，淡水から海水への移行時に鰓の塩類細胞の入れ替わりが盛んになることが報告されているが，本研究では海水に移行した個体よりもむしろ淡水に保持した個体のほうが塩類細胞の入れ替わりが盛んであった．この結果より，ティラピア卵黄嚢上皮の塩類細胞は成魚の鰓に比べて機能の可塑性に富んでいる可能性が示唆された．

第4章　ティラピア仔魚の淡水適応時におけるナトリウムイオンの取り込み機構

淡水中で成魚は鰓からイオンを積極的に取り込むことが出来る．Ca^{2+}は塩類細胞から取り込まれるとされているが，Na^+については取り込み部位の同定はなされておらず，鰓が未発達な発育初期におけるイオン取り込み機構に至ってはほとんど知見が得られていない．そこでまず，ティラピア受精卵を淡水および海水に保持し，仔魚期における体内イオン含量を測定したところ，両群とも餌を与えていないにもかかわらず，発育の進行に伴ってNa^+，Ca^{2+}含量が増加することが明らかとなった．この結果は，胚・仔魚が淡水中からNa^+とCa^{2+}を積極的に取り込んでいることを示しており，これらのイオンの取り込み部位は機能的な鰓を持たない発育初期においては卵黄嚢上皮や体表皮であると考えられる．成魚の鰓においてNa^+は，液胞性H^+-ATPase (V-ATPase) によって排出されたH^+と相補的に，Na^+ channelを通して環境水から細胞内に取り込まれると考えられている．そこでティラピア仔魚について，V-ATPaseに対する特異抗体を用いて光顕および電顕レベルの免疫染色を行ったところ，V-ATPaseは淡水群の仔魚の卵黄嚢上皮の被蓋細胞頂部に局在し，塩類細胞には存在しないことが明らかになった．また，海水に適応している仔魚からはV-ATPaseは検出されなかった．これらの結果から，ティラピアの発育初期においては，淡水からのNa^+の取り込み部位は塩類細胞ではなく被蓋細胞であると考えられる．また，淡水群と海水群の

仔魚について被蓋細胞の密度を計測したところ，淡水群が海水群よりも有意に高かった．細胞数の多さは淡水中における活性やturnover rateの高さを反映していると考えられるので，ガス交換の場である被蓋細胞がイオン調節にも関与している可能性が強く示唆された．

本研究のまとめ

1）ヒラメの変態期における低塩分耐性の獲得にはプロラクチン産生能の増加が大きく関わっていると考えられた．またプロラクチンはヒラメの仔稚魚期を通して低塩分適応ホルモンとして働いていることが示唆された．

2）ヒラメ前変態期仔魚は卵黄嚢および体表に多数の塩類細胞を備えているが，変態期にこれらの塩類細胞は消失し，鰓の塩類細胞が発達することが明らかとなった．

3）ティラピア胚の卵黄嚢上皮に存在する塩類細胞は淡水から海水に移行しても消失せず，淡水型から海水型へとその形態と機能を変化させることが同一細胞の追跡観察によって示された．

4）ティラピア仔魚は淡水中でNa^+とCa^{2+}を積極的に取り込んでいることが明らかとなった．またNa^+の取り込み部位は卵黄嚢上皮の被蓋細胞であることが示唆された．

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